

## ANTIBODY-FORMING CELLS.

A.J. Cunningham.

Many of the experiments described in this thesis were prompted by the suggestions of other people. With this proviso, the work was designed and carried out largely by the candidate. The antibody-forming cells of sheep lymphatics (chapter 4) were studied in collaboration with Mr J.B. Smith. Dr E.R. Harvey and his staff were responsible for sectioning and examining electron microscope specimens. The

A thesis submitted for the degree of  
experiment in chapter 5 was done with Dr A. Jaffery.

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### STATEMENT.

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*A.J. Cunningham.*  
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To my parents.

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Vertebrates typically respond to parenteral injection of foreign macromolecules by producing antibodies. These are globulins which may be distinguished from other serum proteins by their property of specific combination with the eliciting antigen. The kinetics of antibody production following immunization with microorganisms or with artificial antigens has been exhaustively described, and this work has suggested practical immunological procedures for preventing or combating natural infections.

## CHAPTER 1.

### INTRODUCTION.

Much less well understood are the cellular events which lead to the appearance of antibody after antigenic stimulation. Even the nature of the antibody-forming cell is not fully agreed upon. This first chapter records what is fairly certain about the cells involved in the antibody response, and attempts to explain why so little is known about them. The antibody-forming cells themselves are the most thoroughly discussed, since they are the chief subjects of the experimental work of this thesis. Because of the enormous literature published on the subject, the following review is highly selective.



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METHODS FOR DETECTING CELLS CONTAINING OR RELEASING  
ANTIBODY.

I. Correlations.

A large number of early studies demonstrated that lymphoid tissue is the main site of antibody formation in the body (reviewed by McMaster, 1961). The first attempts to identify the actual cell types involved relied on indirect evidence. Antibody and gamma-globulin production were related to increased numbers of plasma cells in humans and in rabbits (Bing and Plum, 1937; Björnboe and Gormsen, 1943). Harris et al. (1945) extracted antibody from cells issuing from antigenically stimulated rabbit lymph nodes, and concluded that the active cells were lymphocytes, since these were by far the most common cell type in the lymph. Fagréus (1948) correlated antibody production in rabbits with the appearance of immature plasma cells in their spleens. The impossibility of drawing conclusions from this sort of evidence is exemplified by two papers published at the same time by Erich<sup>h</sup> et al. (1949), and Harris and Harris (1949). These two groups carried out almost identical studies on the response of rabbit lymph nodes to antigenic stimulation: one correlated antibody production with plasma cell formation and the other, with the local generation of new lymphocytes.

## II. Bacterial adherence.

The first direct demonstrations of the cells involved in antibody production were claimed by Reiss et al. (1950) and Moeschlin and Demiral (1952). It was shown that Salmonella bacteria in suspension would adhere specifically to a proportion of cells from the lymph nodes of immunized rabbits. Most of the adherence-positive cells were thought to be plasma cells. Hayes and Dougherty in 1954 showed a similar agglutination phenomenon around 10-20% of the small lymphocytes in loose connective tissue spreads from immunized mice: in retrospect, this agglutination would seem to have been caused largely by free antibody derived from a minority of the cells.

## III. Histochemical Methods.

A widely used method for detecting antigens or antibodies in tissues was developed by Coons and applied to identifying antibody in single cells (Coons et al. 1955; Leduc et al. 1955). In the "sandwich" version of the technique, antigen is allowed to combine with intracellular antibody in sections of tissue. The preparations are washed to remove free antigen, and the specifically bound antigen is then stained with further antibody coupled to a fluorescent dye. When examined in the fluorescence microscope, cells containing anti-

body fluoresce with an intensity which is presumed to be proportional to the amount of antibody they contain. The chief advantages of this technique are that a fairly large number of cells may be screened for activity, and a wide variety of antigens may be used. Its main drawback is that cell morphology is somewhat obscured by fluorescence, although this can be overcome by comparison with conventionally stained adjacent sections. Fluorescence detects content, not production of antibody, and the cells must obviously be killed for the demonstration.

Radioactively-marked antigens have been used by Berenbaum (1958) to detect cells containing antibody which can combine with and localise the antigen. This method is presumably less technically satisfactory than the Coons technique since its use has rarely been reported.

#### IV. Microdrop Methods.

The main advantage of studying single cells in microdrops under oil is that any antibody produced on incubation can be confidently attributed to the isolated cell. Nossal used bacterial immobilization and agglutination, and the adherence of bacteria to cells as an index of specific antibody production. (Nossal, 1958; Nossal and Makela, 1962a).



Living cells could be studied, and rough quantitation of the amount of antibody produced was possible. The method was time-consuming and difficult to apply when the proportion of active cells in a population was very small. However some preselection of cells producing anti-O antibody was possible when a population of cells was incubated with a large number of motile bacteria: the antibody-formers were set in motion by attached bacteria.

A similar method was used by Attardi et al. (1964) to detect cells releasing phage-neutralizing antibody. Single rabbit lymph node cells were incubated for 48 hours in microdrops together with viable test phages. After incubation, the contents of the drops were recovered on filter paper, resuspended in broth and assayed on lawns of appropriate indicator bacteria for unneutralized phage. Since no preselection of antibody-forming cells was possible, the rabbits used in these experiments were hyperimmunized to provide a population of lymph node cells in which many cells were active. Attardi et al. were mainly interested in determining the incidence of cells producing antibodies of 2 specificities from multiply immunized animals. For this purpose, suspensions of both immunizing phages were included in each microdrop, together with an unrelated indicator phage whose assay



provided an estimate of the volume of the drop. In a second series of experiments, the neutralization of phage by incubated single cells took place in much larger but more accurately measured volumes of medium in sealed micropipettes.

The results obtained by microdrop experiments are discussed later.

#### V. Adherence of erythrocytes to cells.

A method similar in principle to bacterial adherence has been used for assaying antibody-forming cells by Zaalberg (1964), and Nota et al. (1964). The lymphoid cells under test are incubated in a tube with red cells of the type used for immunization: clusters of erythrocytes form around those cells with antibody on their surface. These clusters often involve several rows of red cells indicating that actual release of antibody is occurring. They may be counted in a haemocytometer.

This technique seems to have some advantages over methods based on bacterial adherence. Erythrocytes are relatively large, so clusters can be identified at comparatively low magnifications, which means, in turn, that fairly large numbers of cells can be screened for this effect. However the danger of non-specific clumping has been pointed out (Dent and Good, 1965).

For this reason the method would appear to be unreliable for assaying populations containing large numbers of antibody-forming cells since free antibody in the tubes of incubated medium could agglutinate red cells non-specifically.

#### VI. The Jerne-Ingraham Plaque Principle.

Jerne and his collaborators (1963), and Ingraham and Bussard (1964) introduced a simple technique which allows single cells forming haemolytic antibody to be readily detected even when these comprise a very small proportion of the total population of cells examined. In the Jerne technique, lymphoid cells and target erythrocytes are incubated together in a thin film of agar for an hour or more. During incubation, haemolysin diffuses away from antibody-forming cells and attaches to nearby erythrocytes. When complement is added, the sensitised red cells lyse to form a small clear plaque around a central active cell. Ingraham and Bussard add carboxymethyl cellulose to nutrient medium, and incubate cells and complement together in a small volume of the viscous mixture under a coverslip on a slide.

The great advantage of this technique over previous methods is that it has enormously increased the number of lymphoid cells which can be screened for specific antibody production. A whole mouse lymph node, even

an entire spleen, may be incubated on a Jerne-type plate, and the plaque produced by a single antibody-forming cell detected at a glance. In practical terms this means that the antibody-forming cells in lymphoid tissue can be monitored throughout an immune response: studies using the earlier techniques were largely confined to repeatedly immunized animals, at or near the peak of their response. The most serious limitation of the plaque technique is that it has not yet been adapted for use with defined protein antigens. It has been widely used with erythrocytes, and with enterobacterial lipopolysaccharides conjugated to red cells (Halliday and Webb, 1965). A recent report describes plaque lysis of hapten-coated red cells by cells releasing anti-hapten antibody (Merchant and Hraba, 1966). Schwartz and Braun (1965) have used localised bacteriolysis to indicate antibody production by single cells in a method analogous to the plaque haemolysis technique.

It is probable that the Jerne-Ingraham plaque technique in its original form detects only 19S haemolysin, as suggested by Dresser and Wortis(1965), and Sterzl and Riha (1965). The usefulness of the method has been greatly extended by these two groups of workers who showed that plaques due to antibody of low haemolytic efficiency (probably 7S antibody) could be "developed" by adding anti-gamma globulin to the plates.

This modification is further discussed in Chapter 5.

VII. Evidence that these methods detect antibody content or production.

Four main phenomena have been used to detect single cells producing antibody: plaque formation, fluorescence, bacterial immobilization and the adherence of erythrocytes or bacteria to cells. These reactions all show the specificity of antibody. Furthermore, numbers of active cells in immunized tissue rise and fall in much the same way as serum antibody. Plaque formation and bacterial immobilization obviously involve the release of a factor with the specificity and activity of antibody into the medium around an active cell. Fluorescence, on the other hand, measures only a cell's content of antibody. Adherence of bacteria or erythrocytes to a cell may require cell-bound antibody. The property of causing adherence cannot be passively conferred on normal cells by contact with immune serum (Nossal, 1965 ; Zaalberg et al. 1966): this distinguishes adherence due to antibody production from the adherence of erythrocytes mediated by cytophilic antibody (Boyden, 1964).

Evidence that these effects are antibody mediated is strengthened by the observation that antiserum to gamma globulin influences the detection of active cells by all 4 techniques. Attardi et al. (1964) found that



antiglobulin antisera prevented inactivation of phage by cells which were believed to be releasing anti-phage antibody. Nossal et al. (1964) used this inhibitory property of antisera to distinguish cells releasing 19S and 7S antibody. The work of Dresser and Wortis (1965), and Sterzl and Riha (1965) has shown that plaque formation by 7S haemolysin may be inhibited or enhanced by different concentrations of antiglobulin. Finally, the Coons technique is often used to detect single gamma-globulin producing cells by treating tissues directly with fluorescent antiglobulin antisera.

It seems probable that the active cells detected by the microdrop and plaque techniques are actually synthesizing much of the antibody which they release. Attardi et al. (1964), found that more phage-neutralizing antibody appeared in supernatants above a suspension of incubated cells than could be extracted from the cells themselves. Cell suspensions were also shown to incorporate radioisotope-labelled amino acids into gamma globulins and into specifically precipitable antibody. This is in agreement with a number of previous reports (Helmreich et al. 1962, Taliaferro and Taliaferro, 1957), that the intracellular pool of stored antibody is exhausted by about 30 minutes' incubation, after which cultured lymphoid tissue releases newly formed, labelled



antibody. Evidence for in vitro synthesis of antibody by plaque-forming cells is slightly less conclusive. Ingraham and Bussard (1964) found that preincubation of a population of cells with actinomycin or puromycin greatly decreased the number of plaques counted without affecting cell viability. Jerne et al. (1963), in a similar test, were unable to show any definite effect with actinomycin, possibly because of long-lived messenger RNA in the active cells. However, even inhibition of plaque formation by puromycin cannot be regarded as final proof of antibody synthesis within these cells since the release of stored antibody may also depend on active protein synthesis.

#### 1. Problems.

##### (a). Methods of characterization.

Different methods of preparing the same cells for light microscope examination will give very different results. Probably the best type of preparation for lymphoid cells is a smear stained with a Romanowsky stain. A lot of detail can be seen in living cells with phase contrast optics, although the degree of magnification can not be easily increased. These sections display individual cell morphology much less clearly, and in addition they produce a greater number of cells which are dead and thus, on the average, do not contribute to the relative size of the cell masses.

## B. THE MORPHOLOGY OF ANTIBODY-FORMING CELLS.

It is believed by many that the cell type chiefly, if not solely responsible for antibody formation is the plasma cell (for example, Nossal and Makela, 1962a). However, it has been claimed that other cells, notably small lymphocytes, may also show this function (discussed below). One of the main aims of the experimental work of this thesis was to attempt to provide more information on the nature of the antibody-forming cell. The present section discusses rival claims and suggests possible reasons for the existing state of disagreement.

### I. Problems.

#### (a). Methods of characterization.

Different methods of preparing the same cells for light microscope examination will give very different results. Probably the best type of preparation for lymphoid cells is a smear stained with a Romanowsky stain. A lot of detail can be seen in living cells with phase contrast optics, although the degree of basophilia can not be easily assessed. Tissue sections display individual cell morphology much less clearly, and in addition they produce a greater scatter of cell sizes and tend, on the average, to underestimate the relative size of the cell nucleus.

Perhaps the worst, yet most common preparation used for diagnosing cell type in studies on antibody formation is the low magnification view of a tissue section containing tiny brightly fluorescing cells in which it is difficult to distinguish even a nucleus, let alone finer details.

The electron microscope is bound to replace the light microscope for examining intracellular structure. A thorough correlation of the morphology of lymphoid cells as seen by the two techniques would be desirable, using comparisons of alternate thick and thin sections through the same cells. It is worth noting that the extremely thin sections used for electron microscopy can provide little information on the overall size or nucleus/cytoplasm ratio of cells.

(b). Names.

Groups of cells possessing a number of commonly associated characteristics are given a type name. However, morphological features show continuous variation, and the difficulty of classifying "intermediate" types of cell is well recognized. Confusion can arise unless individual workers define their cell types. Disagreements may be more apparent than real: what one would call a plasmablast, another might label "large lymphocyte".

A more insidious property of names is that they may beg the question, or mislead in other ways. For example,

the term "immature plasma cell" denies the possibility that this cell can avoid becoming a mature plasma cell, and even suggests that, like an immature wine, it has not yet reached the peak of its powers.

(c). Argument from structure to function.

It has often been suggested, (Bernhard and Granboulan, 1960), that cells which do not have large amounts of endoplasmic reticulum in their cytoplasm can not be expected to produce much antibody. This sort of ad hoc reasoning seems unjustified in the absence of certain knowledge on the way in which antibody is synthesized and released by cells. Similarly it may be dangerous to extrapolate from studies on plasmacytopoiesis to make predictions about the development of antibody-forming cells: obviously the functional marker is needed.

(d). Differences in animals, antigens and immunization schedules.

Variations of this kind can always be invoked to explain differences in results. It may be significant that most studies on antibody formation have been done with hyperimmunized animals when plasma cells are relatively common (Fagæus, 1948; Leduc et al, 1955; Wissler et al. 1957).



## II. Names used in the literature.

Lymphoid cells are traditionally classified on their appearance in the light microscope. The main morphological features are: overall size, size of the nucleus, intracellular structure, and histochemical demonstration of cell components. Functional criteria, such as uptake of labelled DNA precursors, are sometimes used. In addition, the normal anatomical position of a cell may be described: thus thymocytes are distinguished from small lymphocytes, although the 2 groups look the same.

### (a). Lymphocytes.

These cells predominate in lymphoid tissue. They vary continuously in size from about 5 or 6 $\mu$  to 12 $\mu$  or more in diameter, depending on the animal of origin and the method of preparation. Small, medium and large lymphocytes are usually recognized, small lymphocytes being most common. Schooley and Berman (1960) distinguished small lymphocytes (not more than 7 $\mu$  in diameter) from medium (8 or 9 $\mu$ ) and large more than 9 $\mu$ ), and showed that a frequency distribution of their diameters was unimodal. Moreover, the average size of the cells could be increased by mixing with leucocyte-free blood before smearing. With Leishman's stain, lymphocytes show a small amount of pale blue featureless cytoplasm. Small lymphocytes usually have a nucleus/total apparent area ratio of about 0.8 or more. Larger



lymphocytes generally have relatively more cytoplasm.

(b). Plasma cells and blasts.

According to Downey's "Handbook of Haematology" (1938), the name plasma cell was first used by Waldeyer in 1875. Cajal, in 1890 and Unna in 1891 restricted the term to certain basophilic cells in connective tissue, while Marshalko in 1895 is said to have defined the cells as recognized today. In fact, the "Marshalko-type" plasma cell with its small eccentric nucleus and cartwheel chromatin is probably peculiar to formalin-fixed sections and is rarely seen in smears. Downey says that plasma cells may vary in size from 1-3 times that of a medium lymphocyte. According to the Nomenclature Committee of the 1959 symposium on "Mechanisms of antibody formation" held at Prague, plasma cells have extensive strongly basophilic cytoplasm, a juxtannuclear clearing, and a Golgi apparatus. "Immature" plasma cells have small nucleoli, eccentrically situated nuclei, and relatively low nucleus/plasma ratio.

Few authors have recorded actual measurements of the cells which they described. Fagœus (1948) found that mature plasma cells varied in their greatest diameter from 6-15 $\mu$  with an overall mean diameter of 10 $\mu$ . The average nucleus/cytoplasm ratio (i.e. ratio of apparent area of nucleus to apparent area of the whole cell) was 0.38 (my calculations from the data of Fagœus).

Immature plasma cells were  $11.1\mu$  in average diameter and 0.58 in nucleus/cytoplasm ratio. For "transitional" cells, which were considered to be the precursors of immature plasma cells, the figures were  $15.7\mu$  and 0.64. Balfour, Cooper and Alpen (1965) describe a nucleus/cytoplasm ratio for "typical plasma cells" of 0.4 and distinguish "plasma cells with very eccentric nuclei" (ratio 0.25).

The Nomenclature Committee at Prague (1960) gave the name "haemocytoblast" to a type of cell which is commonly seen early in immune responses: a large rounded cell with fine chromatin structure of the nucleus and nucleoli and strongly basophilic (pyroninophilic) cytoplasm. The name is unfortunate since it suggests that one and the same cell can give rise to any of the cells of the blood or to antibody-forming cells, which may be true but has not been shown. The term was said to include "blast", "lymphoblast", "plasmablast" and the "transitional cells" of Fagæus. It probably also includes the "activated reticulum cell" of Marshall and White (1950).

Other workers have defined their own subdivisions of the plasma cell series. Schooley (1961), working with mice, recognized early and late plasmablasts, proplasmacytes, and early and late plasmacytes, the last 2 having eccentric nuclei identical to those found in

small lymphocytes, while the late plasmacytes had cartwheel chromatin and were rarely observed. Attardi et al. (1964) defined blasts in rabbits as being 15-20 $\mu$  in diameter, immature plasma cells 12-15 $\mu$ , and mature plasma cells only slightly smaller at 8-14 $\mu$ . Nossal (1959), studying Leishman-stained cells from the lymph nodes of rats, defined blasts as large cells with a large oval reddish-purple nucleus which showed a fine fibrillary chromatin pattern, and with scanty pale blue cytoplasm. Plasma cells were all larger than small lymphocytes, and had eccentric nuclei with a perinuclear clear zone in the dark blue cytoplasm. Three types were recognized: plasmablasts resembled blasts but had darker cytoplasm and more dense nuclear chromatin; immature plasma cells were smaller with more cytoplasm; mature plasma cells were smaller still with very dense or clumped nuclear chromatin, and dark blue cytoplasm which was less abundant than in immature plasma cells.

### III. Antibody-forming cells: Light microscopy.

The classic studies of Fagæus (1948) have already been quoted. She established a firm correlation between antibody formation and the development of plasma cells in the spleens of rabbits. However, techniques were not available at that time for direct demonstration of the active cell types. Pictures published by the workers



who first used bacterial adherence as the criterion of antibody formation (Reiss et al. 1950, Moeschlin and Demiral, 1952, <sup>Hayes +</sup> Dougherty ~~and White~~, 1954), were not adequate for morphological diagnoses. The first unequivocal demonstration that plasma cells are important in antibody formation was provided by Coons and his colleagues (Coons et al. 1955; Leduc et al. 1955). In the lymph nodes of rabbits, groups of fluorescent cells were found which were recognised as mature plasma cells by comparison with adjacent Giemsa-stained sections. The first cells to show fluorescence during an immune response were large, with a big nucleus and basophilic cytoplasm. The possibility that some small lymphocytes contained antibody could not be excluded however. Similar populations of fluorescing cells were described by Vazquez (1961), and by Baney et al. (1962), although both these groups demonstrated a small proportion of fluorescent cells which appeared to be small lymphocytes.

Nossal (1959) has consistently found that rat lymph node cells which release antibacterial antibody are plasma cells of varying degrees of maturity. Small lymphocytes were tested for activity, but were negative in all but one or two cases.

Of the direct evidence that small lymphocytes can, in some situations, release antibody, that of Attardi et al.



(1964) is the most convincing. In their hyperimmunized rabbits, about 70-80% of the lymph node cells were lymphocytic in type, and an average of 10-20% were plasmacytic. Approximately 30% of the plasma cells tested produced phage-neutralizing antibody: active plasma cells classified as immature or mature were about equally common. Approximately 12% of the lymphocytic cells examined released antibody, and these were all small lymphocytes. There was some intentional pre-selection for plasma cells in the single cell experiments, but overall, more than two-thirds of the antibody-formers in the suspensions of lymph node cells were small lymphocytes. Some blast cells were also found to produce antibody. Attardi et al examined living cells with phase contrast optics, and their published pictures support their claim that "optical conditions were very good for phase microscopy".

Other groups have come to the conclusion that plasma cells are not the only cells able to produce antibody. The work of Balfour, Cooper and Alpen (1965) and Balfour, Cooper and Meek (1965) will be discussed later: they describe blasts, small lymphocyte-like cells, and intermediate cells as giving positive fluorescence. Wissler et al. (1957) concluded that the main antibody-former in rats was a large pyroninophilic cell. However, this cell, and the intermediate cell of Balfour<sup>et al</sup>, may well

correspond to Nossal's plasmablasts and immature plasma cells. Recent reports by Van Furth et al. (1966a, 1966b) describe the detection by fluorescence microscopy of cells containing IgG, and IgA or IgM gamma-globulin in humans. In spleens and lymphoid tissue, plasma cells and large and medium lymphocytes were found which contained any one of the 3 types of globulin, while fluorescence-positive small lymphocytes only contained IgM macroglobulin.

#### IV. Antibody-forming cells: electron microscopy.

The first electron microscope examination of antibody-forming cells was reported by de Petris et al in 1963 (and later in 1965). These workers immunized rabbits with ferritin, then cut thick sections of frozen lymph nodes and treated them with the antigen in such a way that the ferritin was able to penetrate into cells through breaks in the cell membranes and become specifically bound to antibody. Thin sections were then made for electron microscopy. In a secondary response, antibody was first detected in blasts 48 hours after immunization. These cells had little endoplasmic reticulum but contained many clustered ribosomes. Later in the response, label could be found in cells with progressively more endoplasmic reticulum and eventually in mature plasma cells. Antibody appeared

to be localized in the cisternae of the endoplasmic reticulum or in the perinuclear space.

Bussard and Binet (1965) cut thin sections through individual rabbit cells found at the centre of 12 separate Jerne-type plaques. In 7 cases, the plaque-forming cell was identified as a plasma cell, and in 1 case, 2 plasma cells were found. Two of the remaining 4 plaques had a plasma cell and a lymphocyte at their centre, and 2 were produced by unidentified cells. Fitch et al. (1965), have briefly reported a similar study of rat haemolysin producers: most of these plaque-forming cells were said to have morphological characteristics of plasma cells.

A more thorough study of the electron microscope morphology of single plaque-forming cells has recently been carried out by Hummeler et al. (1966) and Harris et al. (1966). Rabbits were immunized with sheep erythrocytes, and active cells were obtained from popliteal lymph nodes, popliteal efferent lymph, thoracic duct lymph and peripheral blood. It was clearly shown that cells with only small amounts of endoplasmic reticulum could form antibody, in confirmation of the work of de Petris<sup>et al (1965)</sup>. Such cells were called lymphocytes, although it was pointed out that there were more of the structures generally associated with protein

synthesis (Golgi bodies, nucleoli and short channels of endoplasmic reticulum) in plaque-forming than in normal lymphocytes. In the lymph nodes, antibody-forming cells fell into 2 classes, lymphocytes and plasma cells, with a range of intermediate forms. Active cells from lymph and blood were all small and spherical with an approximately central nucleus which retained condensations of chromatin considered to be characteristic of lymphocytes. The cytoplasm of these free-floating cells showed a profusion of polyribosomes and variable amounts of endoplasmic reticulum. Antibody-forming cells from peripheral blood had parallel rings of lamellar endoplasmic reticulum around the nucleus. Typical plasma cells were not found in blood and lymph.

#### V. Conclusions.

It is generally agreed that plasma cells can produce antibody. Further, there is considerable morphological heterogeneity among antibody-forming cells, and many of the active cell types described may have been developing towards the mature plasma cell form. The nature of the active small lymphocytes described by Attardi et al., the Harrises and others is not clear. They could be normal small lymphocytes, or perhaps plasma cells which have shed most of their cytoplasm (Thiéry, 1960), or possibly a separate cell line with a distinct developmental



sequence. Two types of investigation might be expected to resolve this problem: (i) better characterization of antibody-forming cells by light and electron microscopy at different stages of the immune response; (ii) studies of the developmental history of antibody-forming cells.

### C. DEVELOPMENTAL HISTORY OF ANTIBODY-FORMING CELLS.

#### I. Problems.

One would like to know what cells are initially stimulated by antigen, how these give rise to antibody-formers, and how this process is controlled and prevented from escalating. The ideal experiment would seem to be to take a cell, stimulate it in vitro with antigen and watch the development of a clone of antibody-forming cells. Such a study has not yet proved technically feasible. The necessity for carrying out experiments in vivo means that the cells under test are swamped in a sea of irrelevant material; even methods for picking out and characterizing active cells can give no direct information on the events taking place before antibody formation begins. Furthermore, when an intact animal is antigenically stimulated, antibody-forming cells probably arise from a number of heterogeneous and asynchronously dividing clones, and the developmental pattern of individual clones is obscured. It is for these reasons that comparatively little is definitely known about the developmental history of antibody-forming cells, while a vast body of conflicting literature testifies to the complexity of the subject.

## II. Methods.

### (a). Sequence of transitional forms.

A method much used by the older histologists involved piecing together hypothetical sequences of cellular differentiation from a series of static pictures of immunized tissue. It could give only suggestive results.

### (b) "Quantitative histology".

This method depends on the counting of numbers of cells in different arbitrarily assigned morphological compartments at different stages of the immune response (Leblonde and Sainte-Marie, 1960; McMillan and Engelbert, 1963). The validity of the results depends on 2 main assumptions, both of which are probably wrong: (i) that all cells which look the same behave the same, and (ii) that only "end cells" leave the pool under examination in significant numbers.

### (c) Independent markers.

The best whole animal studies of cellular events in antibody formation have used a number of different properties to mark cells in an attempt to define distinct subgroups within the whole population of the lymphoid tissue. Investigations using only the functional marker, antibody formation, have analysed the kinetics of appearance and decline of antibody-forming cells

(Jerne et al. 1963; Wigzell et al. 1966). Examination of the morphology of functionally active cells gives additional information (Leduc et al. 1955; Coons et al. 1955). Many workers have used a third marker, incorporation of radioactive isotopes into new DNA (Nossal et al. 1963; Baney et al., 1962). Tritiated thymidine is the most commonly used DNA precursor: some of the pitfalls in its use have been discussed by Lajtha (1959). Finally, a powerful fourth marker has been used by Balfour, Cooper and Meek (1965), who stained active cells with Feulgen stain and measured their DNA content by microdensitometry.

(d). In vitro culture.

A number of recent reports claim to have demonstrated the initiation of a primary response in organ cultures in vitro (Globerson and Auerbach 1965; Saunders and King, 1966; Tao and Uhr, 1966). Bussard (1966) has found that normal mouse peritoneal cells may be induced to form plaques by incubation with erythrocyte antigens in carboxymethyl cellulose gum. Mishell and Dutton (1966) produced an apparent primary response in dispersed cell cultures of normal mouse spleens using red cell antigens and Jerne-type assays for antibody-forming cells. A considerable number of plaque-formers also appeared in their cultures when antigen was not added.



Hannoun and Bussard (1966) have maintained cultures of spleens and lymph nodes from immunized rabbits for more than 3 weeks, and have shown that antibody-forming cells which look like macrophages may develop. It is evident that the abnormal conditions of in vitro culture can grossly alter the morphology of cells. However, these in vitro techniques are potentially of great importance for studying the cellular events in antibody formation, especially if some method can be found to culture homogeneous populations, or better still, single clones.

(e). In vivo culture.

The transfer of more or less defined populations of cells plus antigen to inert (usually X-irradiated) genetically similar hosts is a technique in many ways similar to in vitro culture (Makinodan et al. 1960). Harris et al. (1963) conclusively showed that the antibody produced in one such situation was of donor origin. However, there is always a fear that the host may contribute to an immune response which is supposedly mediated by the transferred cells. Many workers have used experiments of this design to examine cellular developmental histories, often in conjunction with fluorescence microscopy and autoradiographic techniques (Vazquez, 1961; Sainte-Marie and Coons, 1964;

Capalbo et al. 1962; Cohen and Cohn, 1964; Makela and Mitchison, 1965).

(f). Colony formation.

When  $10^6$ - $10^7$  normal spleen cells are injected intravenously into an X-irradiated isologous host together with sheep erythrocytes as antigen, localised areas of antibody-forming tissue appear in the spleen within a few days. (Kennedy et al., 1966; Playfair et al. 1965). A linear relation was demonstrated between the number of spleen cells injected and the number of colonies produced: for this reason, they were believed to represent the progeny of a single cell from the original inoculum. Playfair et al. cut up the host spleens and incubated the pieces on a Jerne-type plate, when regions of erythrocyte lysis appeared around the active areas in the presence of complement. Kennedy and his colleagues sliced frozen spleens and incubated the slices on Jerne plates in the same way, to construct a map of the active regions. Moreover, by parallel assays for plaque-forming cells on the spleens of mice which had received the same number of donor cells, they were able to estimate the average number of antibody-forming cells in a colony.

These colonies differ in 2 important ways from the haemopoietic clones described by Till and McCulloch (1961). Precursors of the latter are much more

numerous in normal spleens, and, on settling in a new host they divide to form a morphologically recognizable nodule of about a million cells. It has been directly demonstrated by Becker et al. (1963), using chromosome markers, that most of the cells in these nodules are members of the one clone. The colonies of Kennedy and Playfair, on the other hand, are not morphologically distinguishable because of their small size, but are functionally defined.

Spleen colonies are further discussed in chapter 7.

### III. The precursor cell.

The identity of the antigen- (or messenger-) sensitive progenitor cell which gives rise to antibody-forming cells, is of great importance to immunological theory. In the primary response, it determines the animal's range of immunological responsiveness, and in the secondary it presumably carries the property of memory (Gowans and McGregor, 1965). Three properties of this cell are considered here: morphology, frequency of DNA synthesis, and range of potential reactivity.

#### (a). Morphology.

There is considerable evidence from the work of Gowans and his colleagues that the small lymphocyte may be the ultimate precursor cell in both primary and

secondary responses. It has been conclusively shown that the cell is not an inert end cell, but can differentiate, in the graft-versus-host situation, into a large pyroninophilic cell, which may then revert to the small lymphocyte form. (Gowans et al. 1962; Ford et al. 1966). However, a direct demonstration that small lymphocytes can give rise to antibody-forming cells is lacking.

Gowans and McGregor have further shown that the ability of X-irradiated rats to mount a primary response to sheep red cells can be restored by injecting suspensions of almost pure small lymphocytes. The possibility of "rescue" of host mechanisms by the injected cells was largely eliminated by the demonstration that small lymphocytes from a tolerant donor did not restore specific responsiveness, although it was not shown that this tolerant population could in fact restore responsiveness to antigens other than sheep red cells. The property of conferring secondary responsiveness to bacteriophage also appeared to be carried by lymphocytes (Ford et al. 1966). A point of difference between the primary and secondary responses was that whereas the ability to react to primary antigenic stimulation was severely depressed in rats depleted of lymphocytes by drainage of cells from chronic thoracic fistulae, such depletion did not affect secondary responsiveness. This could be



interpreted to mean that some "memory" cells are sessile, and not removed by drainage, or that they are free-floating, and that only small numbers need be left in the cannulated animal for a full secondary response to be observed. Makela and Mitchison (1965) have in fact shown, in studies on adoptive transfer of immunity in mice, that the size of a secondary antibody response is independent of the number of primed cells transferred over a wide range. On the other hand, White (1963) has presented evidence that a primed popliteal node responds more vigorously to secondary antigenic stimulation than the unprimed opposite node, suggesting that not all memory cells are able to circulate freely.

Hall and Morris (1964) demonstrated that the capacity of a local lymph node to generate an immune response depends not on a radiosensitive local population of cells, but on a recirculating population of lymphocytes (Gowans and Knight, 1964). Further, the characteristic cells appearing in efferent lymph after antigenic stimulation are produced in the node, probably from the recirculating cells (Hall and Morris, 1965). A large proportion of the recirculating pool must pass through an antigenically stimulated node (Hall and Morris, 1965), and this extensive traffic may ensure an adequate number of contacts between competent cells and antigen-laden macrophages (Gowans and McGregor, 1965).

(b). Frequency of DNA synthesis.

Nossal and his colleagues (Nossal and Makela, 1962b; Makela and Nossal, 1962; Nossal et al, 1963; Mitchell et al. 1963) injected tritiated thymidine into rats 2 hours before a primary or a secondary dose of flagellar antigen in both foot pads. The fact that nearly all plasma cells were labelled several days later suggested that the precursor cells had been synthesizing DNA before contact with antigen. However, Mitchell et al. (1963) have themselves pointed out that interpretation of their experiments was complicated by probable reutilization of label in the lymph nodes.

The only other direct evidence for or against the regular division of precursor cells in the absence of antigenic stimulus comes from work by Cohen and Talmage (1965). Spleen cells from a primed mouse were incubated for 2 hours in vitro with tritiated thymidine, washed, and injected into irradiated recipients, together with antigen. Of 109 antibody-forming cells detected by fluorescence 2 or 3 days later, none was labelled with tritium. There are 3 main objections to concluding from this experiment that the precursor cell is a non-dividing type in the secondary response: (i) conditions in vitro may be suitable for stimulated cells to synthesize DNA, but unsuitable for unstimulated cells: (ii) contact with thymidine for 2 hours might label

only a small proportion of a slowly-dividing cell line; (iii) in the 48 or 72 hours between antigen stimulation and identification of antibody-forming cells for autoradiography, sufficient cell divisions may have taken place to remove all label by dilution.

(c). Range of potential reactivity.

An unsolved problem of immunology is the number of antigens to which a single immunologically competent cell can react. This is technically difficult to test directly, but a number of incidental observations are relevant. First, the demonstration that a single cell may form antibodies of 2 specificities (Attardi et al. 1964; Hiramoto and Hamlin, 1965), has set a lower limit to the competence of antibody-forming cells. Second, the phenomenon of antigenic competition (reviewed by Adler, 1964) suggests that precursor cells may be multipotential, although the interpretation of such complex experimental situations is difficult. Third, the fact that memory is specific points to the existence of committed precursor cells, at least in the secondary response.

Recently, the in vivo cloning method of Till and McCulloch (1961) has been used in direct attempts to study the antibody-forming potential of cells from single clones (Trentin and Fahlberg, 1963; Feldman and Mekori, 1966; Trentin et al. 1966). Lethally irradiated



"primary" recipient mice were injected with  $10^5$  normal bone marrow cells, or with  $4 \times 10^7$  lymph node cells from phytohaemagglutinin-stimulated donors. The large discrete colonies which developed in the spleens of primary recipients were dissected out, and cells from one or several of these colonies were used to restore immunological competence to further lethally irradiated recipients. The fact that nearly all such secondarily repopulated recipients could respond to all antigens tested was taken as evidence for the immunological pluripotentiality of the single cells which initially gave rise to the spleen colonies.

This argument depends entirely on the assumption that spleen colonies represent uncontaminated clones, like bacterial colonies on an agar plate. It seems clear from chromosome marker experiments (Trentin et al. 1966) that the great majority of cells in a colony are of donor type. In any case, immunologically competent host cells are no doubt destroyed by the irradiation. Furthermore, 2 observations suggest that most of the cells in a colony are the progeny of a single cell: first, the "one-hit" statistical relation between the number of cells injected and the number of colonies obtained (Feldman and Mekori, 1966); second, the demonstration by Becker et al. (1963), that rare chromosome abnormalities are present in most of the dividing cells in a



colony. However, it does not follow that all the cells in a colony must be members of the clone. If  $10^5$  bone marrow cells are injected into an irradiated mouse, and  $2 \times 10^4$  reach the spleen (Siminovitch et al. 1963), a small number will divide rapidly to form recognizable colonies, each occupying perhaps 5% of the mass of the spleen. Assuming a random distribution of injected cells throughout the host spleen, 5% of the mon-colony-forming donor cells are likely to be present in each of these nodules. One thousand such "passengers" in a colony of more than  $10^6$  cells would be undetectable by morphological or statistical means. Transfer of this colony now represents not the transfer of 1 clone, but that of 1001 potential clones, since many of these passengers may require appropriate antigenic stimulation before they will proliferate. Trentin et al. (1966) recognize this objection, but consider that few immunologically competent cells would be trapped in a colony. Feldman and Mekori used 400 times as many cells to establish their primary colonies, and the incidence of contaminants would probably have been correspondingly higher in their experiments.

The work of Celada and Wigzell (1966) also suggests that the cells which restore immunological competence may not be members of the clones which form the basis of

morphologically prominent nodules. They find that when sheep red cells are injected into animals which are recovering from mid-lethal irradiation, groups of plaque-forming cells develop both within and outside those nodules which spontaneously appear. Where such plaque-forming cells are found inside a nodule, they represent from  $10^{-6}$  to  $10^{-2}$  of the cells in the nodule. They consider that cells which are not members of the haemopoietic clone may be "seeded in" from outside and may proliferate to give rise to these small groups of antibody-formers after contact with antigen. It is also possible that small numbers of cells capable of reacting to specific antigen may arise by differentiation or mutation within a clone. Trentin et al.<sup>(1966)</sup> themselves report that lymphoid tissue develops diffusely throughout an irradiated spleen (and no doubt through any haemopoietic nodules as well) after an injection of normal bone marrow cells (1966). Such lymphoid cell contaminants seem more likely to be precursors of future antibody-forming cells than do members of the haemopoietic clone.

Although proof is lacking, it may be true that a single bone marrow cell can expand into a large clone-colony, and can, over a period of several weeks, repopulate a new irradiated host with cells competent

to respond to any antigen. This would be no more remarkable than the ability of the fertilized egg to expand its potential in the same way over a similar period of time from conception to the development of an immunologically competent young mouse. In both situations, one would like to know whether the ability of an animal to respond to any antigen is acquired, as has been suggested by Burnet (1966), by numerous mutations among lymphoid cells, or whether this information is all present in the ultimate precursor cell. More immediately, can the immunologically competent cell, the cell which responds to antigenic stimulation by generating antibody-forming progeny, react to all or to a restricted number of antigens? Experiments with the colony-transfer model do not appear to be capable of answering these questions unequivocally.

(d). Conclusions.

The precursor cell in both primary and secondary responses seems likely to be the small lymphocyte, although the evidence is not conclusive. If this morphological identification is correct, it still says nothing about the frequency of division of these cells. Many small lymphocytes are known to have a long life span, (Little et al, 1962; Norman et al. 1965),

but the proportion of cells in any population which is stimulated by a given antigen is so small (probably about 1 in  $10^6$ ) that these may well represent a subgroup with unusual properties. Morphological evidence alone does not exclude the possibility that precursor cells could be rapidly dividing small lymphocytes, and the autoradiographic evidence for the frequency of DNA synthesis by these cells is conflicting. Finally, no definitive experiment has yet been found to test the range of antigens to which a single competent cell can react.

#### IV. Cellular events after stimulation.

Direct assays of lymphoid tissue, for antibody-forming cells rather than for antibody, have proved that a real lag phase may follow antigenic stimulation (Ingraham and Bussard, 1964; Wigzell et al. 1966, chapter 5, this thesis). When erythrocyte antigens are used, numbers of plaque-forming cells increase rapidly in spleens or lymph nodes for 4 or 5 days, then decline rather less sharply (Jerne et al., 1963). A second peak, of 7S plaque-forming cells, can be identified when the antiglobulin assay of Sterzl and Riha (1965) and Dresser and Wortis (1965), is employed (see chapter 5). A feed-back effect of antibody may control the amount of proliferation of antibody-forming cells



which takes place (Finkelstein and Uhr, 1964; Moller and Wigzell, 1965; Rowley and Fitch, 1965). Steiner and Eisen (1966) have suggested that persistent antigen continuously influences the expression of potential for antibody production by cells.

It is well established that antibody-forming cells arise by active mitosis from precursors, and that these cells continue to divide and produce antibody while differentiating (Baney et al. 1962; Sainte-Marie and Coons, 1964; Zlotnick et al. 1962; Nossal, 1962; Cohen and Cohen, 1964). One developmental sequence which seems to take place is: large blast, to immature plasma cell to mature plasma cell (Nossal and Makela, 1962b; Schooley, 1961; Balfour, Cooper and Alpen, 1965). The number of generations involved in this process is not definitely known, and estimates of generation times range upwards from 6 hours (Sainte-Marie and Coons, 1964).

Balfour, Cooper and Meek (1965), have emphasised the complexity of cell kinetics in a lymph node during an immune response, and the difficulty of analysing proliferative events in cells which have not begun to synthesize immunoglobulins. They studied rat popliteal lymph nodes which had been stimulated with diphtheria toxoid. On the second day of a secondary response, animals were injected intravenously with tritiated

thymidine. Forty-five minutes later, one popliteal node was biopsied, and  $5\frac{1}{4}$  hours later still, the other stimulated node was removed. Antibody- or immunoglobulin-containing cells were identified by fluorescence, then stained by the Feulgen method. These active cells were then submitted to autoradiography and finally to microdensitometry. Thus 3 properties were measured in the same cells: antibody or gamma-globulin content, recent synthesis of new DNA, and actual DNA content. From the last 2 properties, the stage of division cycle occupied by cells at the time of sacrifice could be deduced.

In preparations made 45 minutes after the injection of isotope, there was little correlation between overall cell size, nuclear size, the content of antibody or globulins, rate of DNA synthesis and content of DNA. Five and one quarter hours later, a group of labelled diploid antibody-containing cells could be found which were believed to be post-divisional cells. Some of these were plasma cells, some "intermediate" cells, and some blasts. It was thought that the intermediate cells were derived by division from blasts, and that, in view of their diploid DNA content, the fate of most intermediate cells was direct maturation to plasma cells.

It is clear that a study of single clones would greatly simplify the determination of developmental sequences followed by antibody-forming cells. The in vivo colony method of Kennedy et al. (1966) and Playfair et al. (1965), already described, may be a suitable model for such investigations. Kennedy has shown that there are about  $10^3$  specific antigen-sensitive precursor cells in a normal mouse spleen. These cells seem to undergo an average of 4-7 divisions following stimulation with antigen in the host spleen. Why multiplication stops after 7 or 8 days is obscure; it can hardly be due to the small amounts of antibody produced in the host. Possibly proliferation ceases if the mature plasma cell form is reached: a number of investigators have concluded that the mature plasma cell is an end cell with a short life span in the node (Schooley 1961; Makela and Nossal, 1962b).

D. NATURE OF THE ANTIBODY PRODUCED BY SINGLE CELLS.

This final section will briefly discuss evidence for heterogeneity of specificity, molecular weight, and antigenic composition in antibodies from single cells.

I. Heterogeneity of antigenic composition and molecular weight.

Immunoglobulins are divided into 3 major classes (IgG, IgA and IgM) depending on the antigenic nature of their heavy chains ( $\gamma$ ,  $\alpha$  and  $\mu$ ) respectively (Ceppellini et al. 1964). Each heavy chain can be combined with one of 2 kinds of light chain, ( $K$  or  $\lambda$ ). Bernier and Cebra (1965) found that individual human cells usually contained 1 of the 3 types of heavy chain, together with 1 type of light chain, although 2 types of light or heavy chain were occasionally detectable by mixed fluorescence. Mellors and Korngold (1963) reported that the 3 immunoglobulin types appeared in different human cells, although IgG and IgM globulins were sometimes identifiable in the same plasma cell. Similar fluorescence studies convinced Schoenberg et al. (1965) that IgM was produced in "non-phagocytic mononuclears", in the rabbit, while IgG was made by plasma cells, although they felt that the reverse could sometimes hold.



By contrast, Chiappino and Pernis (1964) reported that in the red pulp of human spleens, single cells contained either IgG or IgM globulins separately, and that no consistent morphological difference could be found between the 2 groups of cells. The recently discovered human immunoglobulin, IgD, has also been described as occurring in a separate group of cells, which, like IgM and IgG producers, may be "large lymphocytes", or immature or mature plasma cells. (Pernis et al. 1965).

Individual variants of the main immunoglobulin groups may be recognized serologically as allotypes. Weiler (1965), used enhancement of plaque formation by anti-allotype antiserum to distinguish the allotypes of antibody from single cells. He found that in heterozygous rabbits, individual cells tended to produce only one of the 2 antibody allotypes. Similarly, Cebra et al. (1966) reported that different allotypes of rabbit immunoglobulin heavy chains, identified by fluorescence, were contained in different cells.

The molecular weight of an antibody molecule obviously depends on its structure, and the terms "IgM", and "IgG" are often used as synonyms for "19S" and "7S" gamma globulins. However Kim et al. (1964, 1966) have demonstrated an IgG, 19S globulin in mice and piglets, which suggests that S values should be used to describe

antibodies when sedimentation rate, but not antigenic composition, is known. Direct evidence for heterogeneity in the molecular weight of antibody produced by single cells comes from Nossal et al. (1964).

These workers extracted antibody from individual lymph node cells of rats immunized with Salmonella flagella, and titrated it in microdrops. The antibody was characterised by treatment with mercapto ethanol, and in some cases with a specific rabbit anti-rat 7S globulin serum as well. Of 123 antibody-forming cells, 42 were found to contain 19S antibody, 64 7S and 17 both. The double producers were most common at times when a switch-over from 19S to 7S antibody was occurring in the serum. No consistent morphological difference between the 3 classes was found: almost all the active cells were identified as blasts, immature or mature plasma cells. It was suggested that many cells or clones synthesize first 19S, and later 7S antibody with the same combining sites.

## II. Heterogeneity of antibody specificity.

In different experimental situations, from 0 to 45% of the antibody-forming cells from multiply immunized animals have been found to produce antibodies of 2 specificities. Friedman (1964) examined some thousands

of plaque-forming cells from the spleens of mice immunized with sheep and chicken red cells, using Jerne-type preparations with mixtures of both target erythrocytes in the agar. All plaques were "hazy" and involved lysis of only one of the red cell types: none was clear, as would have been expected around cells releasing both antibodies. Nossal detected 1.8% of double producers among antibody-forming cells from rats immunized with 2 or more non-cross-reacting salmonellae (Nossal and Makela, 1962a). Attardi et al. (1964), described a higher incidence of cells producing antibody to 2 unrelated phages: the exact proportion of doubles calculated depended on arbitrary (although stringent) confidence limits, but was of the order of 10%. Finally, Hiramoto and Hamlin (1965), immunized guinea-pigs with human gamma globulin, then used paired fluorescence to detect cells containing antibody to 1 or both of 2 distinct determinants on the same antigen molecule: 45% of the active cells contained antibodies to both determinants.

One of the main aims of studies of this type has been to estimate the range of antibody-forming potential of single cells from an analysis of their phenotype. In all cases the number of double producers scored has been a minimum estimate. Many of or all of the antibody-forming cells could have been manufacturing antibodies with

specificities undetectable by the tests used. One would like to know how much antigen is "read" (in the instructive or selective sense) by the potential antibody-forming cell, and how the antibody specificities which it produces are distributed among the different molecules released. A more systematic method of investigating this problem is tentatively proposed in chapter 6. different model systems.

(3). To define ways of obtaining direct information about the developmental history of these cells.



E. AIMS OF THESIS.

- (1). To develop new methods, based on the Jerne-Ingraham plaque principle, for detecting and characterizing individual antibody-forming cells.
- (2). Application of these methods to a study of the nature of antibody-forming cells, and the antibody they produce, in different model systems.
- (3). To define ways of obtaining direct information about the developmental history of these cells.

CHAPTER 2.

DETECTING SINGLE ANTIBODY-FORMING CELLS.

The plaque technique of Jerne et al. (1963), and of Ingraham and Bassard (1964) for assaying single antibody-forming cells involve the lysis of erythrocytes adjacent to the active cells in order to reveal the antigen-antibody reaction. Maximum sensitivity with this system would be expected if a single layer of lymphoid cells and target erythrocytes were examined. This chapter describes a method which allows the detection of cells producing antibody sufficient to lyse only 10-20 adjacent erythrocytes.

## CHAPTER 2. TECHNIQUE.

### I. Description.

DETECTING SINGLE ANTIBODY-FORMING CELLS.

A sheet of glass, 2 holes 1 cm in diameter punched out with a microtome, and 2 holes 1 cm in diameter punched near either end of the sheet with a cork borer. This sheet is dried on to a microscope slide forming 2 shallow chambers. A suspension of the lymphoid cells to be examined is made in Eagle's medium at 0°C, and complement and target erythrocytes are added to final concentrations of 10% and 5% respectively. The mixture is warmed to room temperature for a few seconds, and a

\* Preparation of single-cell suspension from lymphoid tissue is described in appendix 2.1.

The plaque techniques of Jerne et al. (1963), and of Ingraham and Bussard (1964) for assaying single antibody-forming cells involve the lysis of erythrocytes suspended in three dimensions around the active cells in a supporting medium, agar or methyl cellulose. Maximum sensitivity with this system would be expected if a single layer of lymphoid cells and target erythrocytes were examined. This chapter describes a method which allows the detection of cells producing antibody sufficient to lyse only 10-20 adjacent erythrocytes.

#### A. THE FREE SUSPENSION TECHNIQUE.

##### I. Description.

A sheet of paraffin wax 2cm x 4cm and 10 $\mu$  thick is cut with a microtome, and 2 holes 1cm in diameter punched near either end of the sheet with a cork borer. This sheet is dried on to a microscope slide forming 2 shallow chambers. A suspension of the lymphoid cells to be examined\* is made in Eagle's medium at 0°C, and complement and target erythrocytes are added to final concentrations of 10% and 6% respectively. The mixture is warmed to room temperature for a few seconds, and a

\* Preparation of single-cell suspension from lymphoid tissue is described in appendix 2.1.

small drop (about 10 $\mu$ l) is pipetted on to the glass at the edge of a chamber. A round coverslip 1.3cm in diameter, is lowered slowly on to the drop, which spreads to fill the chamber and overflows it. Excess fluid is sucked off with filter-paper from around the edges of the coverslip, which settles on to the rim of paraffin beneath, and the chamber is sealed with a heated mixture of equal parts of paraffin wax and "Vaseline". This is most easily applied with an open-ended tube of the same diameter as the coverslip. When the slides are incubated at 37°C, plaques begin to appear in the monolayer around haemolysin-producing cells within 1-2 minutes. A maximum number may be counted after 20 minutes. (Figs. 2.1 - 2.4.)

## II. Quantitation.

### (a). Calculating the proportion of active cells.

The area of each chamber is calculated from 2 measurements of its diameter taken at right angles to one another. Accurate measurements can be made under a stereomicroscope at X10 by holding the slide over squared graph paper. The total number of lymphoid cells in a chamber is estimated by counting these cells in several high power fields of accurately calibrated area. The proportion of plaque-forming



Fig.2.1. A "free suspension" chamber. A circular coverslip has been sealed over the cell monolayer with paraffin-"Vaseline" (heavy black ring). A small proportion of mouse spleen cells is producing plaques by lysis of adjacent lipopolysaccharide-coated sheep erythrocytes. (x5).

Fig. 2.2. Higher magnification of part of a chamber: cells from the lymph node of a mouse immunized with sheep erythrocytes. (x20).

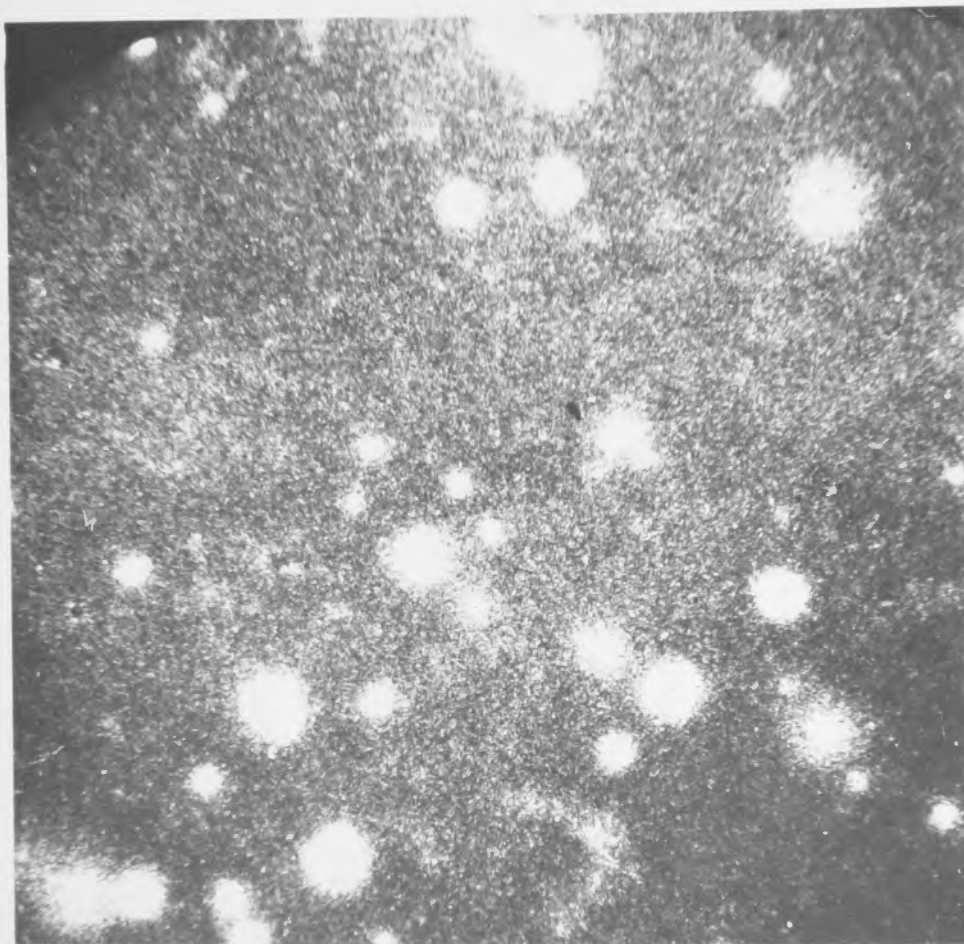
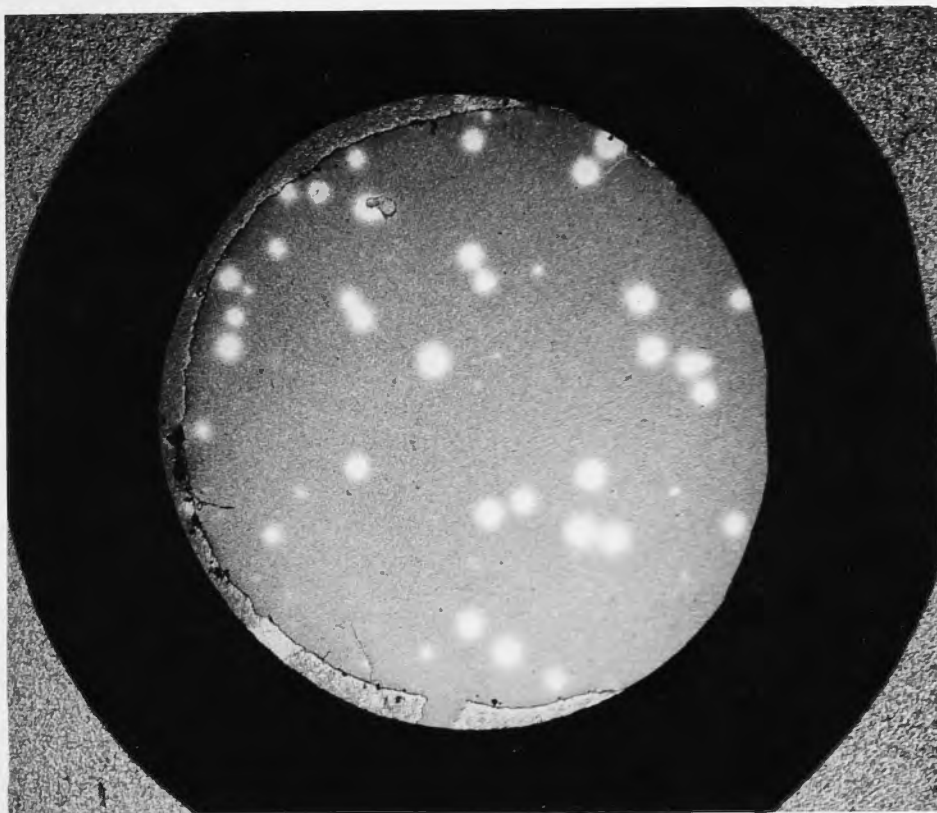


Fig. 2.3. Mouse lymph node cell producing a rather small plaque by lysis of adjacent sheep erythrocytes. Since there are several lymphoid cells near the centre of this plaque, it is not possible to be certain which one is active. (x100 approx).

Fig. 2.4. Phase contrast view of a very small plaque, probably produced by antibody from the large central cell. Sheep red cell ghosts are visible. Inactive nucleated cells may be seen to the left of the plaque. (x500 approx).

cells in the test suspension may now be calculated from the expression

$$N = \frac{7.4 \times 10^4}{A} \times \frac{F}{E} \quad (1)$$

where  $N$  = number

$A$  = area

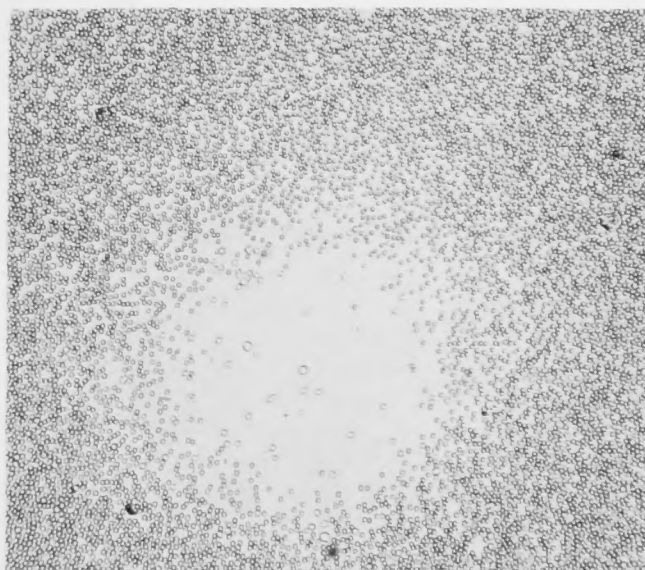
$F$  = number

$E$  = area

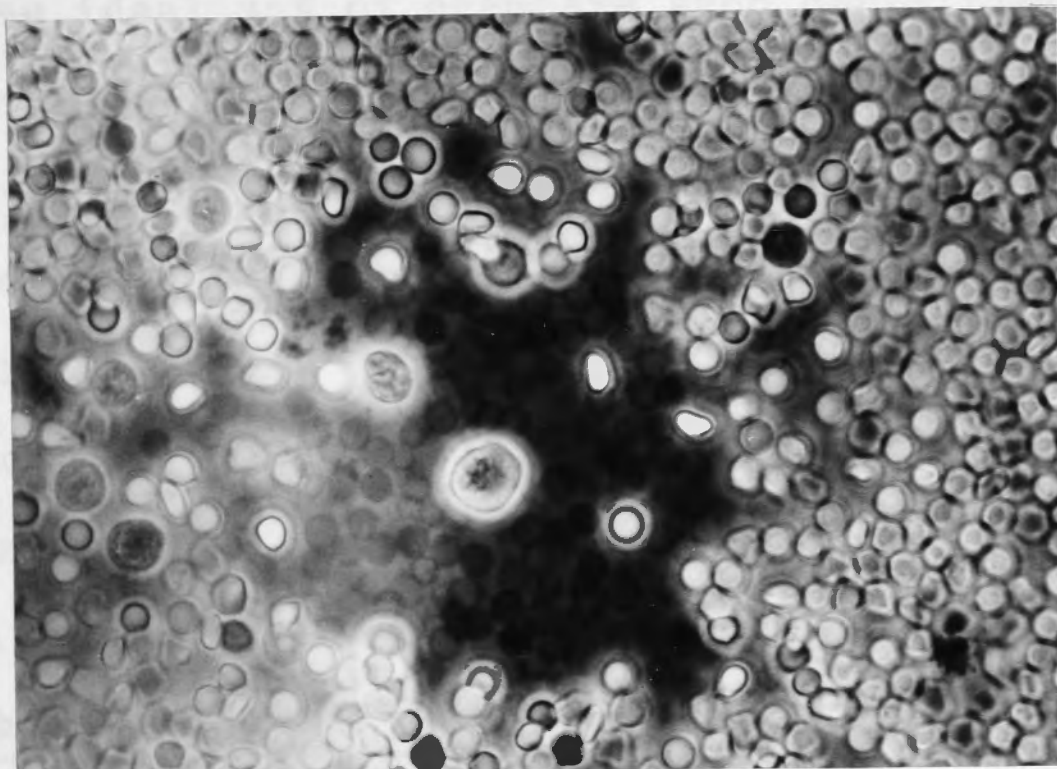
of

Expression (1)

of the 3



determined with an error of less than 1%. The only significant uncertainty in the estimate of  $N$  from a single chamber arises from  $F$  and  $E$ . The number of plaques counted,  $F$ , would vary in Poisson fashion



An experiment was designed to test the correlation



cells in the test suspension may now be calculated from the expression

$$N = \frac{7.4 \times 10^4}{A} \times \frac{P}{H} \dots\dots\dots(1).$$

where N = number of plaque-formers/ $10^6$  lymphoid cells

A = area of the chamber in  $\text{mm}^2$

P = number of plaques in the chamber

H = average number of lymphoid cells in a high power field.

Expression (1) is derived in appendix 1.1.

Of the 3 variables in this expression, A is easily determined with an error of less than 2%. The only significant uncertainty in the estimate of N from a single chamber arises from P and H. The number of plaques counted, P, would vary in Poisson fashion between an ideal set of chambers each containing the same total number of lymphoid cells. Counts of H in a single chamber would also be expected to follow a Poisson distribution if the cells were evenly distributed throughout the chamber. A theoretically derived expression, based on these assumptions, which relates the variance of N to the variance of P and H, is given in appendix 1.2.

(b). Empirical test of error.

An experiment was designed to test the variation

TABLE 2.1

Repeated estimates of the proportion of plaque-forming cells from different samples of the same population.

A = area of chamber in  $\text{mm}^2$ .

N = estimated number of plaque-forming cells/ $10^6$ .

H = lymphoid cells in a high-power field.

Chamber no.	Plaques counted	Area A $\text{mm}^2$	Estimated active cells/ $10^6$ N	Counts of lymphoid cells in high-power fields, H.	Variance/ mean for H.
1	11	85	497	23, 24, 15, 15	1.26
2	17	88	752	23, 23, 16, 15	0.88
3	11	86	902	16, 11, 9, 6	1.69
4	9	93	551	14, 18, 9, 11	1.18
5	14	93	531	20, 16, 24, 24	0.70
6	17	86	790	12, 25, 16, 21	1.75
7	10	90	362	24, 26, 21, 20	0.33
8	8	85	336	26, 17, 22, 18	0.82
9	44	90	720	53, 60, 51, 37	1.84
10	33	85	477	63, 64, 66, 48	1.13
11	36	86	503	66, 77, 33, 70	6.30
12	31	88	507	45, 67, 44, 70	2.20
13	15	86	226	67, 57, 64, 41	2.35
14	17	90	251	58, 60, 57, 48	0.56
15	19	90	411	27, 38, 41, 46	1.70
16	34	86	568	53, 49, 51, 53	0.07
17	24	88	345	60, 62, 56, 56	0.15
18	32	83	610	51, 45, 43, 48	0.25
19	36	93	600	47, 59, 41, 44	1.30
20	30	86	420	62, 61, 60, 54 66, 68, 61, 53, 68, 62.	
Total	448		10,359		26.89
Mean	22.4		518		1.34
Variance			30,170		
S.D.			174		

between repeated estimates of  $N$  from the one population of lymphoid cells. Twenty chambers were prepared, each containing a sample from the same suspension of popliteal lymph node cells of mice which had been injected in each foot-pad with  $10^8$  sheep erythrocytes 5 days previously. For each chamber, plaques were counted and an estimate of  $N$  made. The mean and variance of counts of  $H$  were calculated, and a "best estimate" of the variance of all possible counts of  $H$  for each chamber was made by multiplying the observed variance by  $n/n-1$ , where  $n$  = number of counts of  $H$ . This variance was then divided by the mean of  $H$  as a test of conformity to the Poisson distribution.

Results are shown in table 2.1. The mean of estimates of  $N$  was 518 plaque-forming cells /  $10^6$  total lymphoid cells, with a standard deviation of 174. The mean number of plaques counted per chamber was 22.4. If variation between estimates of  $N$  depended only on Poisson variability in  $P$ , the standard deviation expected would be  $\sqrt{\frac{22.4 \times 100\%}{22.4}} = 21\%$  of the mean, i.e. 109. In fact, it is 1.6 times greater than this. The increased variation is largely due to the error in estimating  $H$ .

The ratio of variance to mean for counts of  $H$

varied between chambers from 0.15 - 6.30 with a mean of 1.34. While low values for this ratio could arise by chance in small samples from a true Poisson distribution, the occasional values much higher than 1 suggest that lymphoid cells are not evenly spread in the chambers. Other similar tests have confirmed this.

In the experiment described here, the numbers of plaques counted in each chamber and the numbers of lymphoid cells per high power field were relatively small. Under optimal conditions, when about 100-150 plaques appear in a chamber containing approximately 100 nucleated cells in each high-power field, the variance of repeated estimates of  $N$  is found to be about 1.3 times as high as would be expected from Poissonian variability in the plaque count alone.

(c). Assigning confidence limits to  $N$ .

One of three procedures was followed:

(i). Thirty to one hundred and fifty plaques were counted per chamber, and about 4 times as many cells in high-power fields. It was then assumed that estimates of  $N$  would be normally distributed with a variance =  $1.5 \times$  Poissonian. So if 100 plaques were counted, confidence limits would be  $\pm 30\%$  of the estimated value of  $N$ .

(ii). Equation (3) from appendix 1.2 was used.



(iii). Where accuracy was critical, experiments were designed with an internal check on the variability of the test.

(d). Other sources of error in the test.

(i). Expelling dissolved gases: The mixture of lymphoid cells, erythrocytes and complement in Eagle's medium was always warmed to room temperature before pipetting into a chamber to prevent the formation of air-bubbles on incubation.

(ii). Filter paper was often used to suck excess fluid from under the coverslip during the "plating" procedure described in section I. Differential counts of the mixture of cells remaining under the coverslip before and after this treatment showed that large cells were not selectively removed.

(iii). The use of heated paraffin-"Vaseline" to seal the test chambers did not appear to damage cells: plaques were no less common at the edges of the chambers adjacent to the sealing mixture than in the centre of the monolayers.

(iv). Occasionally, movement occurred in a monolayer during incubation. This could readily be detected by the distortion of plaques; such chambers were discarded. Slides could be handled quite roughly <sup>without</sup> disturbing the monolayers, provided they were kept approximately horizontal.

(v). Overlap: Chambers were discarded if sufficient plaques developed to cause significant overlapping errors (appendix 1.3). Usually up to 150 plaques could be counted in one chamber. More could be counted if a short period of incubation (15 minutes) was used, since plaques then remained relatively small.

(vi). Counting nucleated cells: For convenience, the lymphoid cells in high-power fields were often counted inside plaques. No difference is expected (or found experimentally) between such counts made within or outside plaques, since the lymphoid cells do not move appreciably after the monolayer forms. However, this procedure involves selecting fields which contain at least one nucleated cell, and could conceivably result in a significant overestimate of the number of lymphoid cells in a chamber if these were present at very low concentrations. Quantitative tests were always done at a lymphoid cell concentration of more than 10 per high-power field, when this effect was negligible. A similar potential source of bias in estimates of the number of relevant lymphoid cells per chamber is the occasional nucleated cell among the sheep erythrocytes. Examination of chambers containing sheep blood cells alone showed that these nucleated cells were rare enough to be ignored.

TABLE 2.2

Effect of washing antibody-forming cells, and of holding them at 0°C before plating.

W= "washed". (2 cycles of centrifugation at 240g).

U= "unwashed".

Washed or Unwashed	Time held before plating (hours)	No. of plaques counted	Estimated no. plaque-forming cells/10 <sup>6</sup> . +95% conf. limits.
U	0.5	45	515 $\pm$ 230
W	0.5	24	378 $\pm$ 231
U	1.0	22	479 $\pm$ 306
W	1.0	37	495 $\pm$ 244
U	1.5	41	639 $\pm$ 299
W	1.5	19	408 $\pm$ 281
U	2.5	18	1120 $\pm$ 795
W	2.5	14	449 $\pm$ 360

TABLE 2.3

Effect of complement concentration on number of plaque-forming cells detected.

Final concentration of complement %	No. of plaques counted	Estimated no. plaque-forming cells/10 <sup>6</sup> .
10	220	561 $\pm$ 113
5	32	506 $\pm$ 268
2.5	7	180 $\pm$ 200 (plaques tiny)
1.25	0	<10

### III. Standardizing conditions of the test.

#### (a). Washing cells, and keeping them at 0°C.

A suspension of spleen cells from a mouse immunized against sheep erythrocytes was divided in half. One half of the cells were washed twice in Eagle's medium by centrifugation at 240g. The others were unwashed. The 2 suspensions were kept at 0°C and assayed at 0.5, 1.0, 1.5, and 2.5 hours after killing the mouse. Table 2.2 shows the results.

The difference between "washed" and "unwashed" groups is not significant at the 5% level, although the tendency towards lower numbers of active cells in the "washed" group may indicate some cell damage due to washing. Cells were usually washed only once, to remove serum haemolysin. Holding cells at 0°C for 2½ hours does not appear to harm them: there is no significant difference between any of the 4 pairs.

#### (b). Concentration of complement.

Table 2.3 shows the effect of complement concentration on the numbers of plaque-forming cells detected in samples from the same suspension of immunized spleen cells. A concentration of 10% was used with the mouse anti-sheep red cell system.

#### (c). Time of incubation of chambers.

Repeated readings on 4 chambers containing mouse



TABLE 2.4

Effect of time of incubation on number of plaques counted.

Time after plating (mins.)	No. of plaques counted in chambers:				Total plaques in the 4 chambers.
	1	2	3	4	
20	20	18	9	13	60
40	20	22	10	13	65
60	23	22	13	11	69
90	20	22	12	12	66
120	21	23	11	11	66
300	19	23	11	11	64

plaque-forming cells are recorded in table 2.4. Nearly all plaques were visible at 20 minutes and a maximum could be counted at 1 hour. This agrees with observations made on the rate of growth of plaques (section IV). Very small plaques sometimes disappeared after prolonged incubation (see section IV for explanation).

(d). Age and origin of red cells.

Red cells from different sheep, and cells kept for varying times at 4°C before use always gave the same results in plaque tests. However, the same batch of erythrocytes was always used for all mice in any one experiment.

(e). Concentration of red cells in chambers.

A final concentration of about 6% red cells in the plated mixture was used. Varying this concentration within fairly wide limits did not affect the number of plaques counted, but it did alter the ease with which plaques were detected. At very low red cell concentrations, plaques were large but indistinct; at high concentrations, plaques were clear-cut, but very small ones tended to be obscured.

(f). Other factors.

Variations in age (6 - 18 weeks) and sex of mice did not significantly affect their ability to produce plaque-forming cells in one small-scale trial.

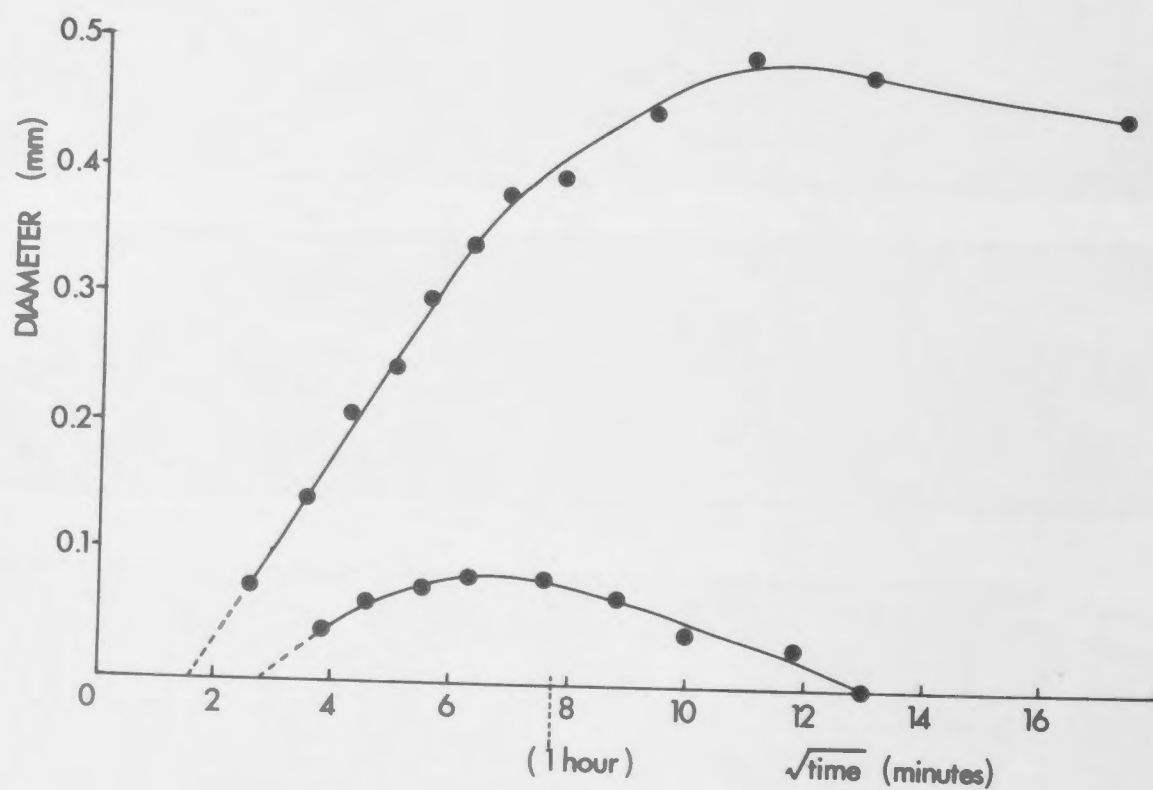


Fig. 2.5. Rate of change in the diameter of 2 plaques incubated at 37°C. The area usually increases linearly with time for the first hour in all but very small plaques. The apparent lag phase of about 4 minutes is probably due to the time taken for slides to reach 37°C.

This was not further investigated since mice of the same age (usually 10 - 14 weeks) and sex were generally used to compare the effects of different treatments on production of antibody-forming cells.

The dose/response and response/time curves in the mouse anti-sheep red cell system are discussed in Chapter 5.

Increasing the size of individual chambers for the free suspension assay is unsatisfactory. The coverslip appears to sag in the centre of large, non-partitioned chambers, and movement of cells by convection is more common than in the standard chambers. The relatively small number of lymphoid cells which can be assayed by the free suspension method (about 300,000 per chamber) remains the chief drawback of the technique.

#### IV. Dynamics of plaque formation.

##### (a). Rate of growth.

Direct proof was obtained that plaques arise by the diffusion of haemolysin away from a central cell when a large number of plaques were observed during formation at 37°C. The area of nearly all plaques increased in roughly linear fashion with time for the first 45 - 90 minutes of incubation (fig. 2.5). This would be expected if antibody is released at a steady rate. (The chambers are three dimensional but their



height, 10-20 $\mu$ , is negligible compared with the diameter of most plaques). A lag period of about 2 minutes as the preparation reached 37°C was seen before this phase of linear growth. After an hour at 37°C, plaques usually stopped growing and often decreased slightly in size, presumably because the surrounding intact erythrocytes, which were always in Brownian movement, met less resistance towards the direction where all their neighbours had lysed. Very small plaques were sometimes completely obliterated after about 2 hours. Many of the nucleated cells in these chambers showed signs of degeneration after several hours at 37°C. They probably ceased to form antibody because of local changes in the concentration of metabolites. Cells incubated in unsealed petri dishes in Eagle's medium kept at a constant pH with CO<sub>2</sub> have been shown to retain their plaque-forming capacity for up to a week (chapter 7.)

(b). Range of sizes.

One of the most striking features of the plaques which are produced by cells from a mouse immunized with sheep erythrocytes is their great range of sizes. The largest grow to almost 1mm in diameter, and the smallest may involve the lysis of only 10 - 20 red cells (fig. 2.2).

No doubt some cells produce undetectable amounts of antibody. The variation in plaque size may be due either to differences in the number of antibody molecules released by different cells or to differences in the lytic efficiency of the antibody. By contrast, when cells from mice immunized with Salmonella lipopolysaccharides are incubated with antigen-sensitised sheep erythrocytes (section B II), the majority of plaques are roughly uniform in size, about 300-400 $\mu$  in diameter (fig. 2.1). One interpretation of this difference between the 2 populations of antibody-forming cells is that lipopolysaccharide antigen gives rise to cells which are nearly all producing approximately the same amount of antibody, while erythrocytes stimulate the appearance of cells releasing much more variable quantities of antibody. This seem unlikely. The difference is probably due to the much greater complexity of the erythrocyte antigens. Many of the cells which give rise to small plaques when incubated amongst sheep erythrocytes are probably releasing antibody which has low haemolytic efficiency because it is directed against some relatively uncommon or inaccessible antigenic determinant on the surface of the erythrocyte.

TABLE 2.5

Comparison of the sensitivity of the "agar plate" and "free suspension" techniques for detecting single antibody-forming cells.

Origin of cells	Hours after primary stimulus	Antigen	Calculated plaque-formers/ $10^6$ nucleated cells			
			Agar plate		Free suspension	
			A*	B*	A	B
Mouse spleen	96	$10^8$ sheep rbc	1090	1030	2880	2760
Mouse spleen	144	$10^8$ sheep rbc	67	40	144	134
Mouse lymph node	120	$10^8$ sheep rbc	232	215	875	950
Sheep efferent lymph	83	$10^9$ boiled <u>Salmonella muenchen</u>	< 10	< 10	11200	10500

\*Plaques were counted by me (A), and by an independent observer (B).

## V. Sensitivity of the method.

Suspensions of cells from the spleens or lymph nodes of mice immunized with sheep erythrocytes were assayed by both "agar plate" and "free suspension" techniques. The results (table 2.5) show that the modified technique is about 3 times more sensitive than the Jerne method. Included in the table is a comparison of assays for cells forming antibacterial antibody from the popliteal lymph of sheep (chapter 4). The very small areas of lysis which these cells produce were undetectable by the Jerne technique and were probably obscured by intact red cells above or below regions of lysis in agar.

## VI. Immuno-cyto-adherence.

The adherence of erythrocytes to lymphoid cells was first used as an index of antibody production by Nota et al. (1964), and by Zaalberg (1964). This phenomenon has been investigated in chapter 4. Immuno-cyto-adherence is readily detected by the standard free suspension technique, both in the presence and absence of complement. The concentration of target erythrocytes must be carefully adjusted: if they are too closely packed, weakly positive lymphoid cells are not detected; if they are too far apart,



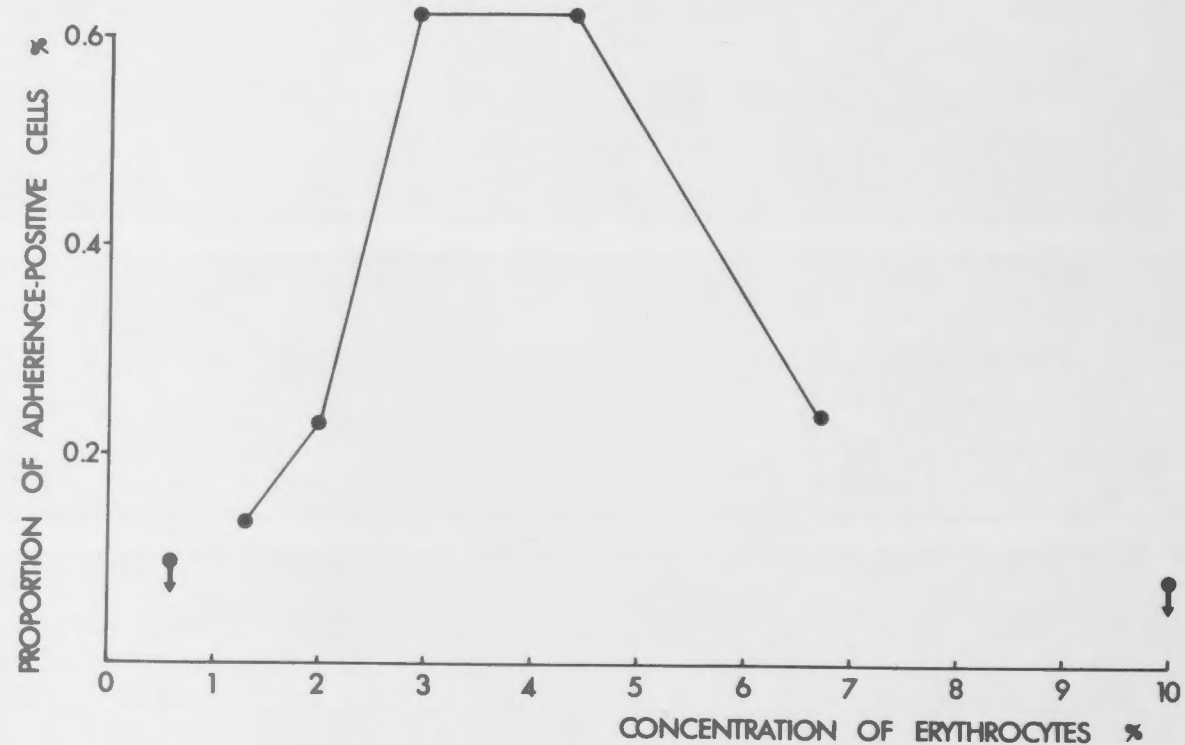


Fig. 2.6. Estimated number of mouse lymphoid cells causing adherence of target sheep erythrocytes when samples from the same population were assayed at different erythrocyte concentrations.

adherence of erythrocytes and lymphoid cells cannot occur. A concentration of 3 - 4% is optimal (fig. 2.6).

## B. APPLYING THE PLAQUE TECHNIQUE TO OTHER SYSTEMS.

### I. Other red cells.

Erythrocytes from 20 vertebrate species were tested for their ability to stimulate production of plaque-forming cells in adult CBA strain mice. Mice were injected intravenously with 0.2 ml of a 1/10 suspension of the test erythrocytes, and their spleens assayed 4 days later. Results are shown in table 2.6. Red cells from sheep, goat, cow, fowl, pigeon, horse, frog, fish, opossum, and echidna gave good results. Rabbit red cells were usually suitable but sometimes failed to provoke the appearance of plaque-forming cells.

A number of these erythrocytes were tested for cross-reactivity with one another (table 2.7). Sheep red cells cross-reacted extensively with goat cells, and to a lesser extent with erythrocytes from a cow. No other strong cross-reactions were found.

### II. Bacterial endotoxins.

Lipopolysaccharide (endotoxin) was extracted from several species of enterobacteria (table 2.8) as described by Halliday and Webb (1965). When red cells

TABLE 2.6

Red blood cells from various species as antigens in the mouse. (Unbracketed figures represent the response of single mice 4 days after primary I/V immunization. Figures in brackets are the approximate mean peak responses of more than 5 mice in different experiments).

Immunizing red cells	Plaque-forming cells/ $10^6$	Approximate maximum plaque size ( $\mu$ ).
Sheep	(1000)	500
Fowl	( 700)	150
Pigeon	(1100)	150
Horse	( 500)	400
Rabbit	( 700)	200
Guinea-pig	< 20, < 20	Only 2 small plaques counted. Only 5 small plaques counted.
Human (grp 0)	< 20	
Goose	20	
Rat	50	
Dog	< 10	130
Cat	100	
Goat	1330	
Kangaroo	< 10	
Echidna	700	400
Cow	840	400
Pig	< 10	150
Frog	370, 340	
Fish (carp)	900	
Opossum	1300	
Mouse (C57/Bl in CBA)	< 10	
Mouse (CBA in C57/Bl)	< 10	

TABLE 2.7

Cross reactions between red cells tested as antigens in CBA mice. (Each figure represents the number of plaque-forming cells/ $10^6$  spleen cells in 1 mouse tested 4 days after immunization. Figures in brackets compare peak responses to the immunizing red cells).

Immun- izing rbcs.	T e s t     r b c s						
	Sheep	Fowl	Pigeon	Horse	Rabbit	Goat	Cow
Sheep	(1000)	<10	<20	<10	<10	430	200
Fowl	<20, <10	(700)	<10	<10	<10		
Pigeon	<20, <10 <10	<20 <10	(1100)	<10	<10		
Horse	<10, <10 <10	<10	<10	(500)	<10		
Rabbit	<20, <10	<20	<10	<10	(700)		
Goat	530					(1330)	350
Cow	100					100	(840)



TABLE 2.8.

Plaque-forming cells produced in response to single injections of isologous red cells sensitised with lipopolysaccharide from 1 of 5 species of enterobacteria.

(Each figure is the no. of plaque-forming cells per  $10^6$  spleen cells in 1 mouse 4 or 5 days after injection).

Antigen (lipopolysaccharide)	4 days	5 days
<u>Salmonella muenchen</u>	90,150,340,180,88, 100,77,160.	550,30
<u>E. coli</u>	420, 15,20	5,20
<u>Shigella sonnei</u>	120,100	50
<u>Shigella flexneri</u>	15	45
<u>Proteus vulgaris</u>	40	45

were incubated with this extract, they became coated with the lipopolysaccharide and susceptible to lysis with immune serum. Mice were immunized with isologous red cells coated with the lipopolysaccharide (Moller, 1965). Four or five days after immunization their spleens contained antibody-forming cells which were able to produce plaques when incubated in a monolayer of sheep erythrocytes sensitised with the antigen. By contrast, mice injected with boiled bacteria or with free lipopolysaccharide responded very poorly. Table 2.8 shows the response of mice to antigens from several species of bacteria.

In order to estimate how much of the available antigen was adsorbed to the surface of isologous red cells, and so injected into the immunized mice, an assay for free lipopolysaccharide was developed using inhibition of the lysis of sensitised sheep red cells by immune rabbit serum. It was found that a 10% suspension of mouse erythrocytes in saline adsorbed approximately one half of the available lipopolysaccharide over a wide range of concentrations when the two were incubated together for 1 hour at 37°C. As an upper limit, 0.1ml of packed mouse erythrocytes could adsorb 50µgm of the crude lipopolysaccharide. Table 2.9 shows the response of mice to different

TABLE 2.9.

Response of mice to isologous red cells coated with different amounts of lipopolysaccharide from Salmonella muenchen.

(Each figure from pooled spleens of 3 mice assayed 5 days after primary I/V injection. Each mouse received 0.05ml rbes with a variable amount of conjugated lipopolysaccharide).

Concentration of crude lipopolysacc. in sensitising solution ( $\mu$ gm/ml)	Estimated total amount of lipopolysacc. received per mouse ( $\mu$ gm)	Plaques counted	Estimated plaque- forming cells/ $10^6$ .
200	25	150	640
100	25	155	920
50	12.5	20	63
25	6.3	12	18
12.5	3.1	17	56
6.3	1.6	2	5
3.1	0.8	2	10

doses of lipopolysaccharide from S. muenchen. There is a rapidly decreasing effect with amounts of antigen less than 25 $\mu$ gm. This may explain the poor response of mice to the lipopolysaccharides of some other enterobacteria in preliminary experiments (table 2.8).

### III. Sheep lymphocytes as antigens.

Cells from the spleens of mice immunized by intravenous injection of approximately  $10^8$  sheep lymphocytes were incubated in standard chambers, with the sheep lymphocytes replacing erythrocytes as "target" cells in the monolayers. Complement and trypan blue were present in the mixture. A search was made for plaques of blue-stained lymphocytes killed by cytotoxic antibody from individual mouse cells, but none was found.

### IV. Other antigens.

Unsuccessful attempts were made to use the plaque technique in mice with a variety of target proteins conjugated to sheep red cells. One reason for this lack of success may have been the poor antibody response of mice to the injected proteins. Also unfruitful were attempts to detect antiprotein antibody released from single cells by the local



TABLE 2.10.

Plaque-forming cells from the spleens of adult fowls at various times after I/V injection of  $3 \times 10^9$  sheep red cells.

(Each figure represents the mean response of 2 fowls).

Days after injection of sheep rbcs.	Plaques counted	Estimated plaque-forming cells/ $10^6$ .
0	2	5
2	1	3
4	105	300
6	30	180
8	8	62
10	7	36

precipitation of antigens labelled with  $I_{131}$ . Cells from immunized mice were incubated in a film of agar on a slide as in the agar drop method of Sterzl and Mandel (1964). Heavily labelled antigen was applied for varying times, then the preparations were washed to remove free antigen. The agar was dried to a thin film and stripping film was applied in the hope of detecting any labelled antigen retained in areas where antibody had been produced. Lack of success may have been due to washing out of very small precipitates from the agar.

#### V. Animals other than the mouse.

##### (a). Fowl.

Cells from adult fowls intravenously injected with  $3 \times 10^9$  sheep red cells produced small plaques (mostly less than  $200\mu$  diameter) in one experiment. The time course of the response (table 2.10) was much the same as for mice reacting to sheep erythrocytes. (chapter 5).

Chick embryos were injected at 11 days of incubation with sheep erythrocytes ( $2.5 \times 10^5$ , or  $5 \times 10^6$ , or  $10^8$ , in 0.1ml). Spleens were assayed 5 and 7 days later, when no plaque-forming cells were found.

(b). Sheep.

The response of sheep lymph nodes to enterobacterial antigens is described in chapter 4. Plaque-forming cells have been found in the efferent lymph from popliteal nodes of sheep stimulated with fowl red cells, but not when horse red cells were used as antigen. Morris (personal communication) found that repeated injections of human erythrocytes did not give rise to plaque-forming cells in the sheep.

least one nucleated cell near their centre; red cell ghosts fill the space; no foreign material can be seen in the plaque.

(b). About three times as many plaque-forming cells are counted with the free suspension method as are seen with the agar-plate technique. More important, the very small (30 - 40  $\mu$  diameter) plaques produced by sheep lymph cells releasing antibacterial antibody were found to be undetectable by the Jerns technique.

(c). Localised Agglutination of erythrocytes around some antibody-producing lymphoid cells may also be detected in the standard chambers (see chapter 4).

The principal disadvantage of the technique is that not more than about 300,000 lymphoid cells may be incubated in one chamber.

### C. DISCUSSION.

The "free suspension" modification of the Jerne plaque technique has three main advantages:

(a). All cells in a monolayer are unobscured, and may be examined at high magnification. True plaques (as opposed to such artefacts as the clear areas produced by air bubbles or pieces of dust), satisfy the following criteria: They are circular with at least one nucleated cell near their centre; red cell ghosts fill the area; no foreign material can be seen in the plaque.

(b). About three times as many mouse haemolysin-producing cells are counted with the free suspension method as are seen with the agar-plate technique. More important, the very small (30 - 40 $\mu$  diameter) plaques produced by sheep lymph cells releasing antibacterial antibody were found to be undetectable by the Jerne technique.

(c). Localised agglutination of erythrocytes around some antibody-producing lymphoid cells may also be detected in the standard chambers (see chapter 4).

The principal disadvantage of the technique is that not more than about 300,000 lymphoid cells may be incubated in one chamber.



The examination of an organ such as the spleen for its content of plaque-forming cells involves the preparation of a single cell suspension, when a proportion of the cells are destroyed. In any such test for activity of individuals from a population of cells, it can be said that the conditions of preparation and testing may affect some types of active cell selectively. For example, a class of antibody-forming cells may exist which is particularly sensitive to centrifugation. Another group may produce antibody of a type which is not detected by the plaque test. This problem of possible selectivity in the assay seems unavoidable. The best that can be done is always to examine cells in a standard way.

The accuracy of the plaque technique has been examined empirically and theoretically. It depends on the number of plaques counted, and on the accuracy of the estimate of the total number of lymphoid cells plated. The latter improves, but with diminishing returns, as the cells in more high-power fields are scored. An efficient compromise is to count about four times as many lymphoid cells as plaques, when the variance of repeated estimates of the proportion of cells forming plaques is about one and one-half times as high as would be expected from Poissonian error in

the plaque count alone. If 100 plaques are counted, 95% confidence limits are about  $\pm 30\%$ .

Antigens which may be used in the plaque test include a range of erythrocytes and bacterial lipopolysaccharides. The test has not yet been adapted for use with defined protein antigens. One difficulty may be that when proteins are conjugated to red cells, an antigen-antibody reaction involving the protein takes place at too great a distance from the cell surface to initiate lysis. However, Hyslop and Roeder, (1966) have been able to attach proteins to red cells in such a way that the red cells can be lysed by anti-protein antibody. These sensitised erythrocytes may prove suitable for use in the plaque test. Merchant and Hraba (1966) have recently succeeded in detecting cells releasing antibody to haptenes by lysis of hapten-coated erythrocytes. The use of haptenic antigens should enable the specificity of the antibody released by plaque-forming cells to be much more precisely defined.

D. SUMMARY.

The standardization of a modified plaque technique for detecting single antibody-forming cells is described. A range of erythrocyte and bacterial antigens has been defined for use with this test in assaying antibody-forming cells from mice and sheep. The technique will detect cells releasing antibody sufficient to lyse only 10-20 erythrocytes.

TECHNIQUE FOR STUDYING THE MORPHOLOGY OF

INDIVIDUAL ANTIBODY-FORMING CELLS.

In the previous chapter, a sensitive technique was described for detecting single antibody-forming cells. These antibody-producers always form a very small proportion of the lymphoid cells from an immunized animal. Nothing can be learned of their morphology by examining the population as a whole; the active cells must be identified by a functional test then examined individually. This chapter describes new methods for characterizing antibody-forming cells by light and electron microscopy. In subsequent chapters, the

### CHAPTER 3.

#### METHODS FOR STUDYING THE MORPHOLOGY OF

#### INDIVIDUAL ANTIBODY-FORMING CELLS.

Staining cells embedded in agar in Jerns-type plates gave very poor morphological definition. Moreover, it was found impossible to spear such cells satisfactorily after cutting them out of the agar. Cells in the standard assay chambers were inaccessible because of the coverslip used to seal the preparations. However, micromanipulation of individual active cells was possible when monolayers of lymphoid cells and erythrocytes were incubated in microdrops under oil.

A thin strip of vinyl plastic was painted around the edges of a microscope slide to form a shallow chamber,



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#### A. LIGHT MICROSCOPY.

Staining cells embedded in agar in Jerne-type plates gave very poor morphological definition. Moreover, it was found impossible to smear such cells satisfactorily after cutting them out of the agar. Cells in the standard assay chambers were inaccessible because of the coverslip used to seal the preparations. However, micromanipulation of individual active cells was possible when monolayers of lymphoid cells and erythrocytes were incubated in microdrops under oil.

A thin strip of vinyl plastic was painted around the edges of a microscope slide to form a shallow chamber,

which was then filled with paraffin oil. Droplets of Eagle's medium containing the lymphoid cells to be tested together with target erythrocytes and 10% complement were blown on to the glass under the oil with a fine pipette. These droplets were usually 1 - 5  $\mu$ l in volume and contained several thousand lymphoid cells. Stable plaques appeared in the red cell monolayer around those cells producing lytic antibody after incubation of the oil chambers at 37°C for 10 - 30 minutes.

Plaque-forming cells were now isolated using a micropipette about 40  $\mu$  in diameter at the mouth, attached to an improvised micromanipulator. Observed at X100, an active cell was blown clear of its neighbours with a stream of medium from the pipette, then sucked up and transferred to a cell-free collection drop. Several such cells were usually collected in one drop. A small volume of serum was then sucked into the micropipette, and the isolated cells drawn into this serum. The serum was blown gently on to a clean slide while the slide was moved backwards and forwards with the stage manipulators of the microscope. The preparation was then stained with Leishman's stain, and the cells found by following the serum trail.

At the beginning or end of an immune response, plaque-forming cells were so rare that it was necessary to plate out lymphoid cells in high concentration in the reaction mixture to find any plaques at all. This resulted in a large number of nucleated cells being present in each plaque. To identify the active cell from among these, a second plating step was used. All the lymphoid cells from a plaque were sucked out, and diluted with more medium plus complement and erythrocytes, in several fresh droplets. These were then reincubated, when the single plaque-forming cell could usually be identified. Using this 2-step procedure, antibody-forming cells could be isolated and smeared when they were as rare as 1 in  $10^6$  of the population.

The method described here demonstrates the morphology of individual cells as clearly as a standard smear (fig. 5.5). However the technique is time-consuming, and the "2-step" process particularly so, which means that relatively few cells can be examined. A proportion of cells is lost during handling: with practice, about three-quarters of the antibody-formers collected are found undamaged on the slide. Preparations of this kind are suitable for high-resolution autoradiography (chapter 8).

Evidence that cells other than the antibody-formers are rarely, if ever, smeared by mistake comes from the experiments described in chapter 4. In all the populations of sheep cells from which individual antibody-formers were selected, the predominant cell type was the medium lymphocyte. An inactive cell, isolated by accident, would most probably have been a medium lymphocyte. However, nearly 200 active cells were individually smeared and stained, and no medium lymphocytes were found among them.



## B. ELECTRON MICROSCOPY.

In selecting cells for electron microscopy, immuno-cyto-adherence was used as the criterion of antibody production in place of plaque formation. The technique was devised using sheep lymph cells (further dsicussed in chapter4).

Individual lymphoid cells with erythrocytes adhering were transferred to a collection drop. All samples were taken at the peak of immune responses when 20-40 of the antibody-forming cells could be collected in less than an hour. Rabbit anti-sheep red cell serum was then instilled into the collection drop, and the cell clusters were gently blown together with the micropipette, to form a single clump under the agglutinating influence of the rabbit antiserum. This clump could then be manipulated free-hand with a fine pipette under a dissecting microscope.

The clump was fixed in glutaraldehyde (2.5% in collidine buffer) for 3 hours, washed twice in Millonig's phosphate buffer then transferred to osmium tetroxide (1% in Millonig's buffer) for 3 hours. Two washes in osmium tetroxide in Palade's buffer followed to remove phosphate, after which the cells were stained for 30 minutes in uranyl acetate (1% in aqueous solution), and dehydrated in alcohol.

This rather complicated alternation of buffers was empirically found to give best staining of the intracellular membranes. Volumes of all reagents were kept small (about 0.1ml in a small tube) to avoid losing the sample. Embedding was done initially in a small drop of araldite in a plastic "boat". After hardening this initial drop, the boat was filled with araldite. Thick sections were cut until the lymphoid cells could be seen between the red cell markers. Thin sections were then cut, and overstained with lead hydroxide.

The cells are never exposed to temperatures greater than 37°C before fixation, and appear to be undamaged by the rabbit antiserum used in this technique. Plaque formation has not been used as a criterion of antibody release since it was thought that the complement required might have damaged the cells to which rabbit anti-sheep antibody was attached. However, cells which produce plaques in the presence of complement are able to agglutinate the surrounding sensitised erythrocytes when transferred to microdrops lacking complement (chapter 4). Plaque-forming cells could no doubt be used if they were washed before transfer to the collection drop.

The principle of clumping together a number of active cells before fixing and embedding greatly simplifies the subsequent cutting of sections.

However, the idea is probably unworkable where less than 1 cell in  $10^4$  of a population is active; it would take so long to collect 20 antibody-formers that the first would have degenerated long before the last was isolated! In situations like this, single cells could be sectioned as described by other authors (e.g. Harris et al. 1966).

ANTIBODY FORMATION BY SINGLE CELLS FROM EMBRYO

ROUTES AND EFFICIENCY OF ANTIBODY FORMATION

In chapters 2 and 3, methods for the detection and characterization of single antibody-forming cells were developed. This section describes the application of these methods to a comparative study of the cells producing antibody to *Salmonella typhimurium* lipopolysaccharide in lymph nodes and efferent lymph of sheep. The sheep was chosen as an experimental animal because a technique exists for cannulating the efferent popliteal lymph duct and collecting lymph for several weeks; (Hall and Morris, 1964); the output of antibody-forming cells from a single stimulated node could thus be estimated.

#### CHAPTER 4.

### ANTIBODY FORMATION BY SINGLE CELLS FROM LYMPH

#### NODES AND EFFERENT LYMPH OF SHEEP.

Evidence is presented that plaque formation and immune-cytoxicity are induced in efferent lymph by release, by active lymphoid cells, of antibody directed against antigens on the surface of surrounding sensitized erythrocytes. Also, changes in cellular response in the popliteal lymph node and efferent lymph with time are described.



In chapters 2 and 3, methods for the detection and characterization of single antibody-forming cells were developed. This section describes the application of these methods to a comparative study of the cells producing antibody to Salmonella lipopolysaccharides in lymph nodes and efferent lymph of sheep. The sheep was chosen as an experimental animal because a technique exists for cannulating the efferent popliteal lymph duct and collecting lymph continuously for several weeks; (Hall and Morris, 1964); the output of antibody-forming cells from a single stimulated node could thus be studied.

Evidence is presented that plaque formation and immuno-cyto-adherence in this system represent the release, by active lymphoid cells, of antibody directed against antigens on the surface of surrounding sensitised erythrocytes. Also, changes in cellular response in the popliteal lymph node and efferent lymph with time are described.

A. MATERIALS AND METHODS.

(a). Animals.

Merino ewes and wethers,  $1\frac{1}{2}$  to 3 years old, were used.

(b). Surgery.

Cannulation of the efferent duct of the popliteal lymph node was done as described by Hall and Morris (1962).

(c). Immunization.

A suspension of  $10^8$ ,  $5 \times 10^8$  or  $10^9$  boiled Salmonella muenchen in 1ml of saline was injected subcutaneously in the posterolateral aspect of the metatarsal region. For the study of efferent lymph cells the lymph duct was cannulated 2-4 days before the node was stimulated. Five primary responses were followed. Secondary injections were made 7-10 days later in 2 of these sheep; the other 3 preparations stopped flowing before the secondary response was completed. Two other sheep were injected 5 weeks before cannulation, and the secondary response studied following a second injection of antigen after cannulation.

Popliteal lymph node cells were obtained from a separate group of sheep whose lymph ducts had not been cannulated. Nine nodes were removed from sheep

1-8 days after primary stimulation with  $10^9$  bacteria. Six further nodes were removed following 2 injections of antigen, 5 weeks apart.

Observations on the cells of afferent lymph have been made in 2 sheep. Both were given a subcutaneous primary injection of  $5 \times 10^8$  boiled bacteria in Freund's adjuvant in the lateral metatarsus; incomplete adjuvant was used in one sheep, complete in the other. Seven days later, afferent lymph ducts were cannulated to examine lymph travelling from the induced granulomata towards the popliteal node. Thirteen days after the primary injection each sheep received a secondary stimulus of bacteria without adjuvant.

(d). Lymph cells.

Lymph was collected in sterile 120ml polythene bottles. Every 12 hours, the volume of lymph was measured, a 15ml sample was centrifuged, and the cells obtained were resuspended in their own volume of normal sheep serum, then smeared on 2 glass slides. Smears were stained with Leishman's stain, and a differential count made of 1000 cells.

Approximately 1ml of lymph was obtained direct from the cannula every 12 or 24 hours for assaying antibody-producing cells. These cells were gently centrifuged out, washed once and resuspended in

Eagle's medium at 0°C. Lymph nodes were cut up with scissors, the fragments pipetted vigorously, and the resulting suspension of cells washed once.

(e) Sensitising erythrocytes.

Washed sheep red blood cells in 0.9% NaCl solution were incubated for one hour at 37°C with bacterial lipopolysaccharide prepared by the method of Halliday and Webb (1965) (100µg lipopolysaccharide/0.1ml packed red cells).

(f). Complement.

A batch of guinea-pig serum was absorbed with one-tenth its volume of sensitised sheep red cells to remove antibacterial antibody, then stored at -20°C.

(g). Assay for antibody-producing cells.

The free suspension modification of the plaque technique was used, as described in chapter 2. Two chambers were also incubated in each test without complement, and with the concentration of sensitised erythrocytes reduced to about 3%. Counts of lymphoid cells with adherent red cells were made at X240 magnification. Up to 5,000 cells were examined in order to record at least 20 positive cells. However, at the beginning and end of a response, when the proportion of active to inactive cells was less than 1/1000, it was not possible to estimate accurately the



number of cells causing adherence.

(h). Examination of active cells by light microscopy.

Single cells were smeared and stained as described in chapter 3. An estimate of the areas of nucleus and cytoplasm of many of the cells smeared in this way was obtained by tracing their projected images on paper (x3,500), then cutting out and weighing the paper.

(i). Electron microscopy of antibody-forming cells.

As described in chapter 3.

(j). Lymph and serum antibody titrations.

See appendix 2.2.

(b) & (c). Adherence and agglutination.

B. RESULTS.

I. The recognition of cells producing specific antibody.

Three types of interaction occurred between antibody-producing lymphoid cells and the monolayer of sensitised erythrocytes surrounding them: (a) plaque formation, due to red cell lysis, occurring only in the presence of complement; (b) immuno-cyto-adherence, similar to the phenomenon observed by Nota et al. (1964) and Zaalberg (1964) in populations of rabbit and mouse lymphoid cells producing antibody to foreign erythrocytes; (c) localised agglutination, involving agglutination of the erythrocytes surrounding, but not

attached to a central lymphoid cell.

(a). Plaque formation.

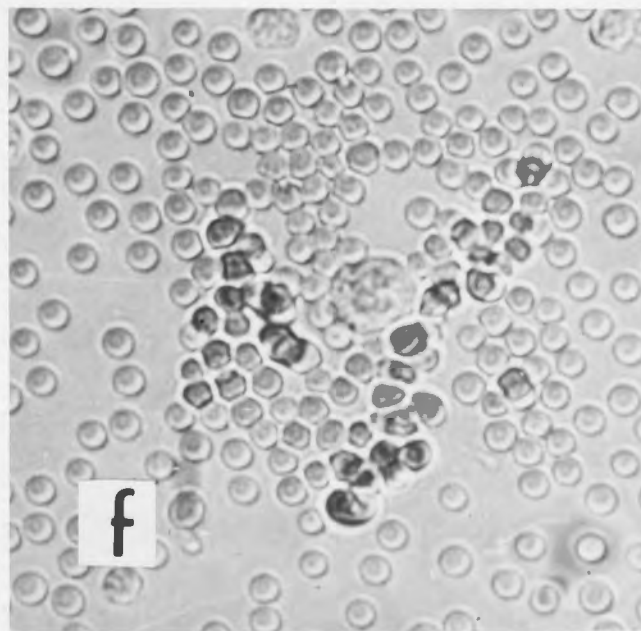
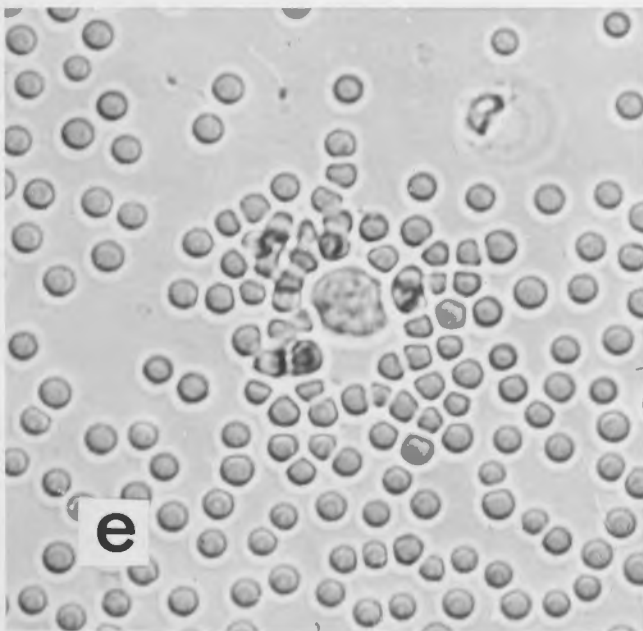
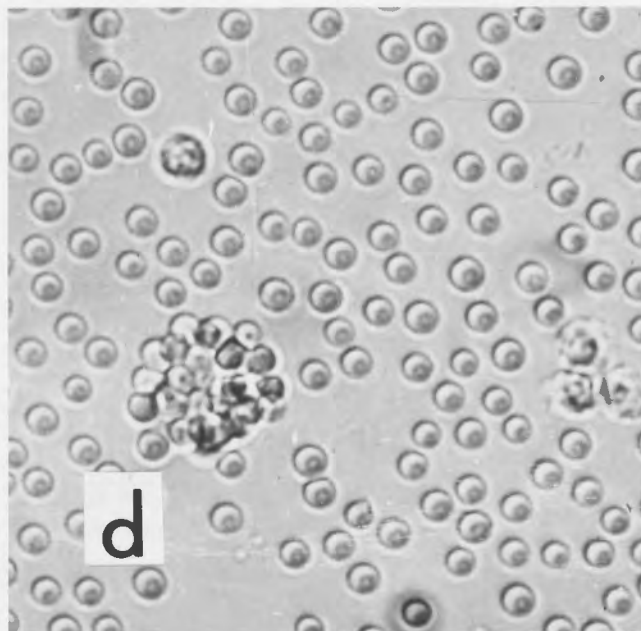
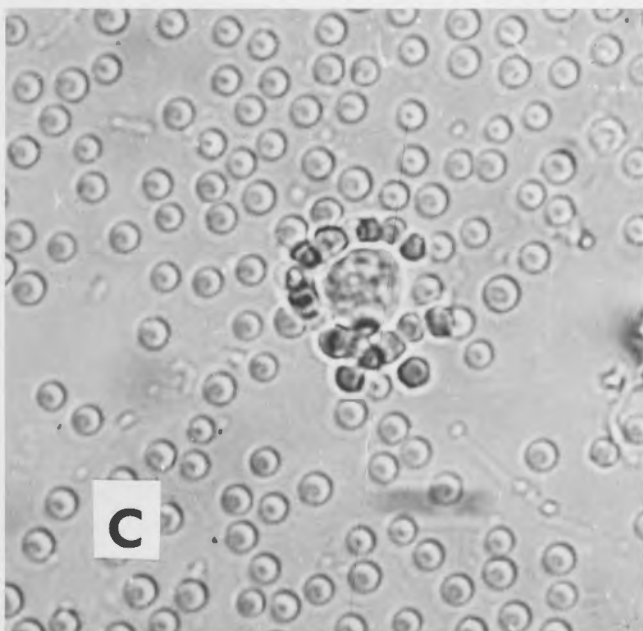
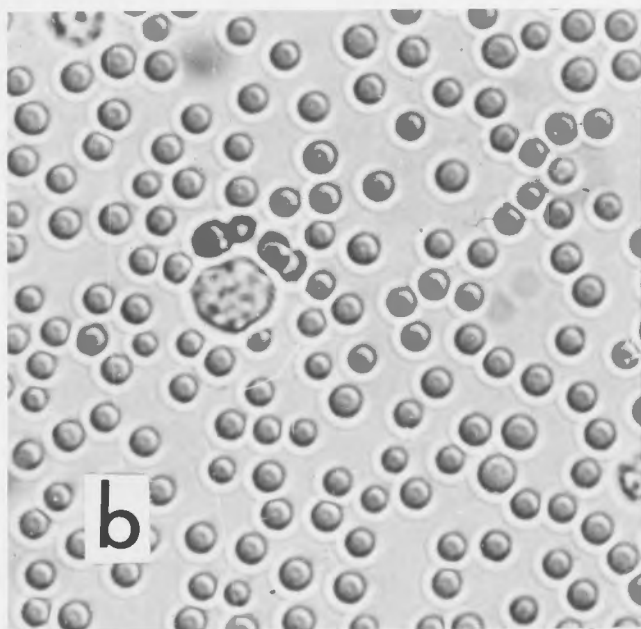
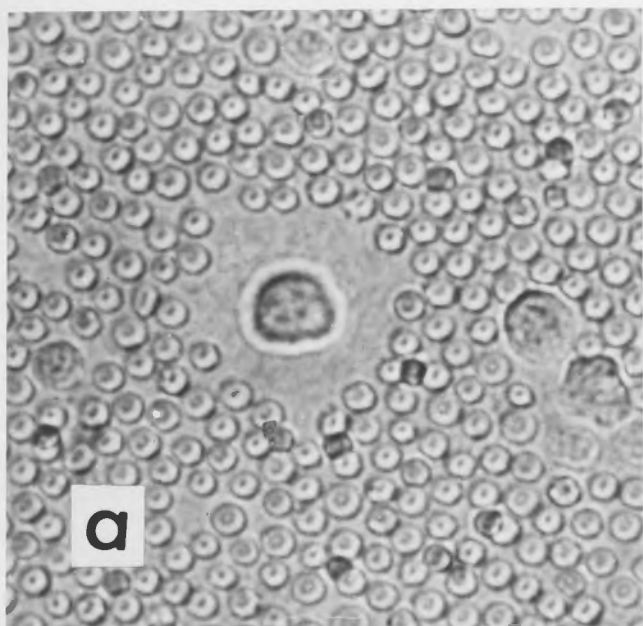
Plaques began to appear in the red cell monolayer after a few minute's incubation, and were usually counted after 20 minutes. Most of the plaques in this system were very small, about 30-60 $\mu$  in diameter, with lysis of only 1-3 'rows' of red cells adjacent to the central lymph cell (fig. 4.1a). A small proportion were up to 300 $\mu$  in size. Larger plaques were more commonly produced by cells from the lymph node than by cells from the lymph.

(b) & (c). Adherence and agglutination.

In the absence of complement, sensitised erythrocytes adhered to a proportion of lymphoid cells from immunized sheep. This reaction varied in extent from the adherence of 5 erythrocytes, (arbitrarily chosen as the smallest positive effect), to the agglutination of all red cells in an area 200 $\mu$  or more in diameter (fig. 4.1). Cells producing adherence were more common than those producing agglutination.

Cells forming plaques in the presence of complement were always able to produce agglutination or adherence of surrounding erythrocytes when transferred to drops containing no complement. However

Fig. 4.1. Effects caused by antibody-forming cells.  
(a) plaque formation; (b), (c), (d), immuno-cyto-  
adherence; (e), (f), localised agglutination of  
sensitised erythrocytes.





the reverse was not always true: many cells showing adherence in the absence of complement were unable to lyse these erythrocytes when complement was added. So at any time, a count of adherence-positive cells gave the highest estimate of the number of antibody-producing cells in the population. The proportion of the total detectable antibody-forming cells which produced lysis varied from one sheep to another (from 1/6 to nearly 1/1), but it was approximately constant for any one immune response. Figs. 4.2 and 4.3 show the parallel rise and fall of plaque-forming cells and total antibody-forming cells in a primary and a secondary response.

II. Evidence that plaque formation, immuno-cyto-adherence and localised agglutination are due to the production of specific antibody.

(a) Mediation by antibody.

(i). These effects all show antibody specificity. Some plaque formation and adherence occurred when lipopolysaccharides from other salmonellae (sharing antigens with S.muenchen) were used to coat the test erythrocytes, but no active cells were seen when E. coli or Shigella sonnei lipopolysaccharides or unsensitised red cells, were used (see chapter 6).

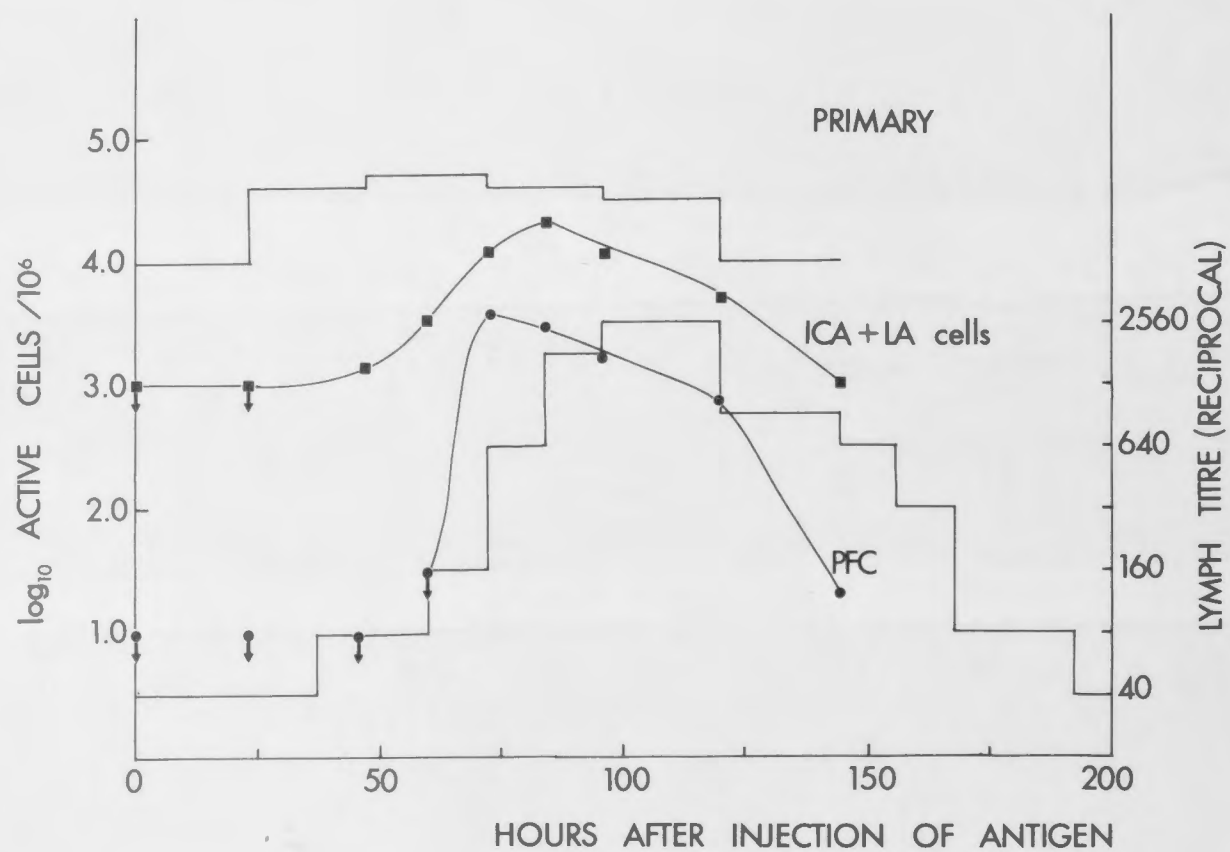


Fig. 4.2.

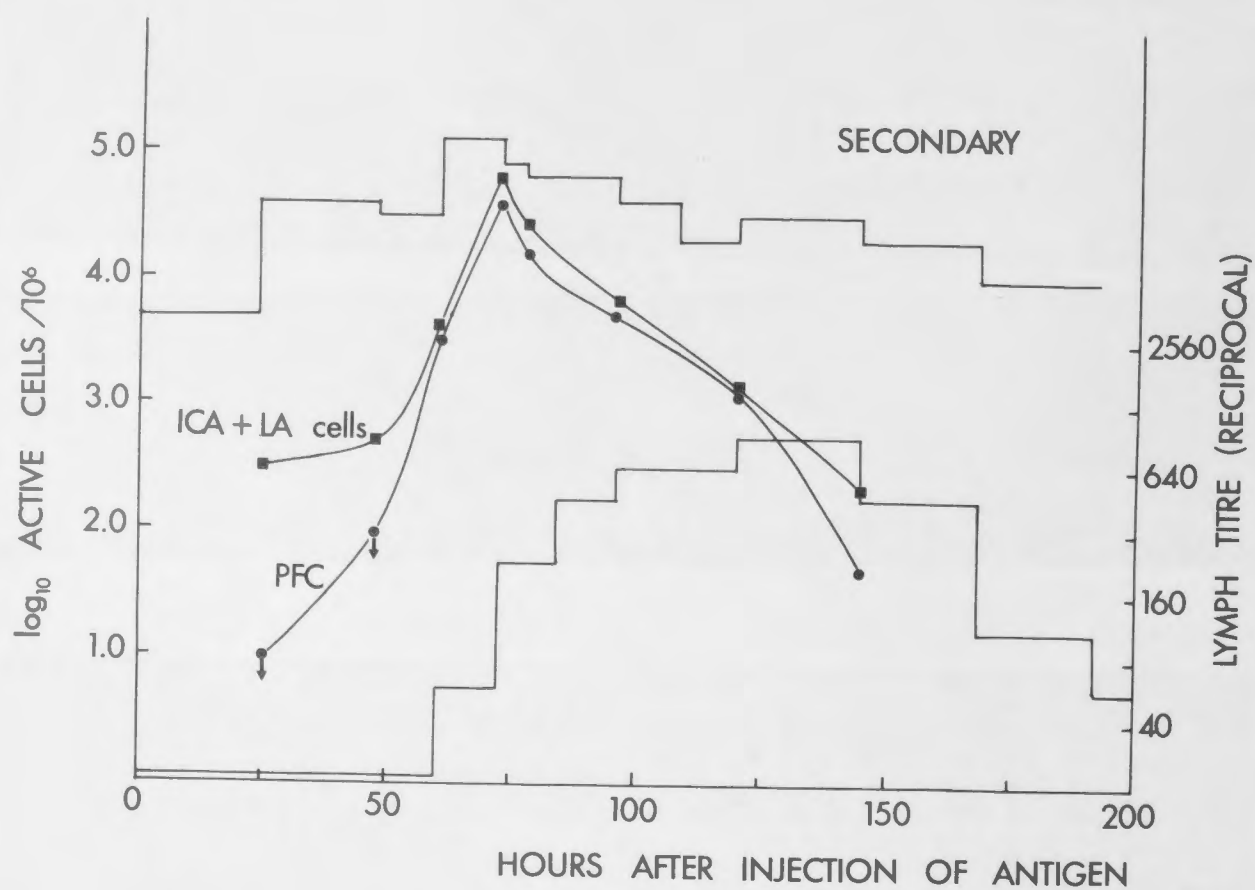


Fig. 4.3.

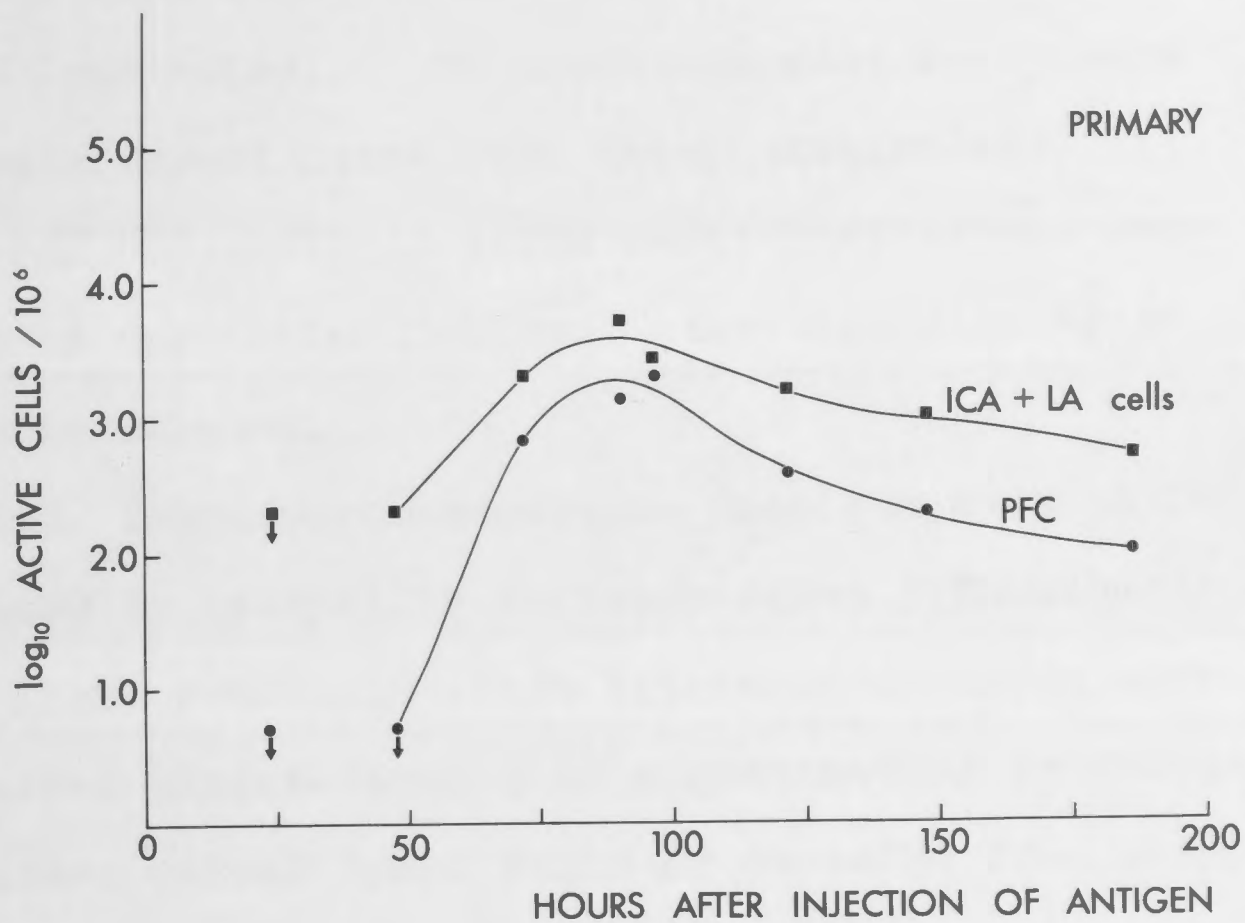


Fig. 4.4.

Figs. 4.2. and 4.3. Changes in the efferent lymph after antigenic stimulation of sheep 3 (primary response) and sheep 4 (secondary). Plaque-forming cells (PFC), and cells showing immuno-cyto-adherence (ICA), or localised agglutination (LA) are plotted as the logarithm of the estimated number per million lymph cells. The upper bar-graph represents the logarithm of the number of basophilic cells per million, and the lower, the lymph haemagglutinin titres.

Fig. 4.4. Changes in the lymph node after antigenic stimulation.

(ii). The numbers of active cells rose and fell about 2 days ahead of antibody titres in the efferent lymph (see below). No plaque-forming cells were found in the efferent lymph from sheep unstimulated by antigen. Occasional cells showing adherence were found in unstimulated sheep; the significance of these is unknown.

(iii). Immuno-cyto-adherence does not seem to be mediated by cytophilic antibody since preincubation with lymph containing high titres of antibody never conferred plaque-forming or agglutinating properties on either normal lymph cells or on cells from sheep responding to an injection of Influenza virus. Immune serum and the supernatant from antibody-producing lymph cells were also ineffective in coating normal lymphoid cells with antibody. In some preparations where a large proportion of the lymphoid cells were producing antibody, incubation of lymphoid cells and target erythrocytes in the same tube, as in the method of Zaalberg (1964), gave rise to massive agglutination of the erythrocytes, and nearly all the nucleated cells were non-specifically caught up in these clumps. For this reason cells producing agglutination or adherence were always counted following incubation in a chamber, where



TABLE 4.1.

Inhibition of immuno-cyto-adherence by free lipopolysaccharide reversed by incubation.

ICA = immuno-cyto-adherence.  
LA = localised agglutination.

Cells prewashed in lipopolysacc.	0°C.			After 10 min. at 37°C.		
	Positive ICA	cells LA	Total counted	Positive ICA	cells LA	Total counted
Homologous	2*	0	3,300	12	5	2,300
Heterologous	14	0	3,200	14	12	2,400

\* these two "positives" were very weak.

they were kept relatively immobile.

(b). Active synthesis of antibody.

Several observations suggest that the cells responsible for plaque formation, immuno-cyto-adherence or localised agglutination are actually synthesizing antibody, although the evidence is not conclusive.

(i). Metabolic activity of the cell is necessary for plaque formation. No plaques appear at 0°C, but they begin to form as soon as the temperature of the chamber is raised to 37°C. Immuno-cyto-adherence can occur at 0°C, but it is inhibited by prewashing the cells in "homologous" lipopolysaccharide, as shown in the following experiment:

Two samples of efferent lymph cells from a sheep immunized with S. muenchen were washed once at 0°C in either the "homologous" lipopolysaccharide (200 µgm/ml) or a Shigella sonnei extract ("heterologous"), at the same concentration. Each sample was then plated out in free suspension chambers with sheep red cells sensitised to S. muenchen lipopolysaccharide, still at 0°C. Cells producing immuno-cyto-adherence or localised agglutination, were counted. The slides were then incubated at 37°C for 10 minutes, and recounted. It can be seen from table 4.1 that incubation at 37°C quickly reverses inhibition of adherence.

TABLE 4.2.

Effect of puromycin on plaque production by lymph cells from sheep immunized with Salmonella muenchen.

Group	Time 0	2 hours ( <u>±</u> puromycin)	3½ hrs (1½ hrs minus puromycin)
"Test"	9700 <u>±</u> 1900	580 <u>±</u> 281	2400 <u>±</u> 1020
"Control"		8000 <u>±</u> 2300	9300 <u>±</u> 2410

(ii). The active cells are all basophilic, suggesting differentiation for protein synthesis (see below). No medium lymphocytes, the predominant cell type in all samples, were identified as causing any of the 3 reactions.

(iii). Cells from an immunized sheep were incubated for 2 hours in medium containing 100  $\mu\text{g}/\text{ml}$  ( $2 \times 10^{-4} \text{M}$ ) puromycin ("test" group). A "control" group was incubated without puromycin. Each group was then assayed for plaque-forming cells by <sup>the</sup> free suspension technique; a sample from the test group was assayed in the presence of puromycin, and 1 from the control, in its absence. The remaining cells from each group were separately washed twice, and reincubated for 30 minutes. This process of washing and reincubating was repeated twice. Both groups were then reassayed for plaque-forming cells in the absence of puromycin. Table 4.2 shows that the puromycin caused a 10-20-fold drop in the number of antibody-forming cells detectable. This effect was partially reversed by a further 1½ hours incubation in medium free of puromycin.

(iv). Lymph cells from an immunized sheep were cultured at a concentration of  $10^7$  cells/ml in petri dishes in Eagle's medium containing 10% sheep serum. Initially about 1700 cells/million were forming lytic



**TABLE 4.3.**

*Peak Numbers of Antibody-Producing Cells and Antibody Titers in Efferent Lymph during the Immune Response of the Popliteal Node*

Sheep	Response	Peak Nos. of plaque-forming cells (active cells/10 <sup>6</sup> )	Peak Nos. of cells causing immuno-cyto-adherence or localized agglutination (active cells/10 <sup>6</sup> )	Peak percentage large basophilic cells	Peak lymph agglutination titers (reciprocal)
3	1*	4,000	21,000	5	2560
4	2	40,000	63,000	12	1280
5	1	12,000	20,000	15	640
5	2	25,000	30,000	29	N†
6	2	11,000	11,000	18	640
7	1	25,000	28,000	11	N

\* 1, primary response; and 2, secondary response.

† N, not measured.

antibody. The numbers fell off, until after 7 days, approximately 300 cells per million of those remaining produced plaques when tested. The cells became very fragile in culture and most of them broke up when centrifuged. Similarly, the antibody-producing cells remaining at 7 days invariably disintegrated when smeared. While these late plaque-forming cells may not have been the same as those initially detected, it is obvious that the ability to form plaques persists for a long time in a cell population, suggesting active synthesis of antibody by a proportion of the cells.

### III. Kinetics of cellular and antibody responses.

#### (a). Efferent lymph and node.

Figs. 4.2 and 4.3 show the changing numbers of active cells in efferent lymph during a primary and a secondary response, each followed in a single sheep. Fig. 4.4 shows the primary response of popliteal lymph nodes from a number of sheep at various times after antigenic stimulation. In table 4.3 the number of active cells present in the efferent lymph, lymph antibody titres, and the proportion of basophilic cells in the differential counts at the peak of 6 responses, are recorded.

No antibody-forming cells were found in either the node or efferent lymph until 40-50 hours after the injection of antigen. After this time there was a rapid increase in the numbers of active cells, which reached a peak at 70-105 hours. The numbers of these cells then declined more slowly, and 8-9 days after stimulation there were usually no active cells in the lymph, although a few could still be found in the node. Lymph agglutinating and lytic titres were always similar, reaching a maximum 30-70 hours after the numbers of active cells reached their peak. Plasma titres were much lower, reaching a plateau about 100 hours after the injection of antigen. No consistent difference emerged between primary and secondary responses in either efferent lymph or lymph nodes. It is quite probable that all the sheep had previously experienced Salmonella antigens, so that all responses were in fact secondary in nature.

Changes in properties of different morphologically defined cell types in efferent lymph during each response were similar to those described by Hall and Morris (1963).

(b). Afferent lymph.

Samples of lymph from the cannulated popliteal afferent ducts of 2 sheep were assayed 1, 2, 3 and 4

days after secondary antigenic stimulation. In addition, lymph from one of the sheep was examined just prior to secondary stimulation, 13 days after primary immunization. No antibody-forming cells were found in any of these samples, in spite of the fact that many basophilic cells were present in the lymph flowing towards the popliteal node from the granulomata induced by the Freund's adjuvant in the primary injections.

(c). Nature of the antibody.

Samples of lymph collected at the peak of each response were treated with 2-mercaptoethanol and retitrated. Samples were also fractionated by density-gradient ultracentrifugation. All but a trace of the agglutinating activity was associated with mercaptoethanol-sensitive antibody of relatively high sedimentation constant (19S) in all responses except one, a secondary response, where about 10% of the antibody had a relatively low rate of sedimentation.



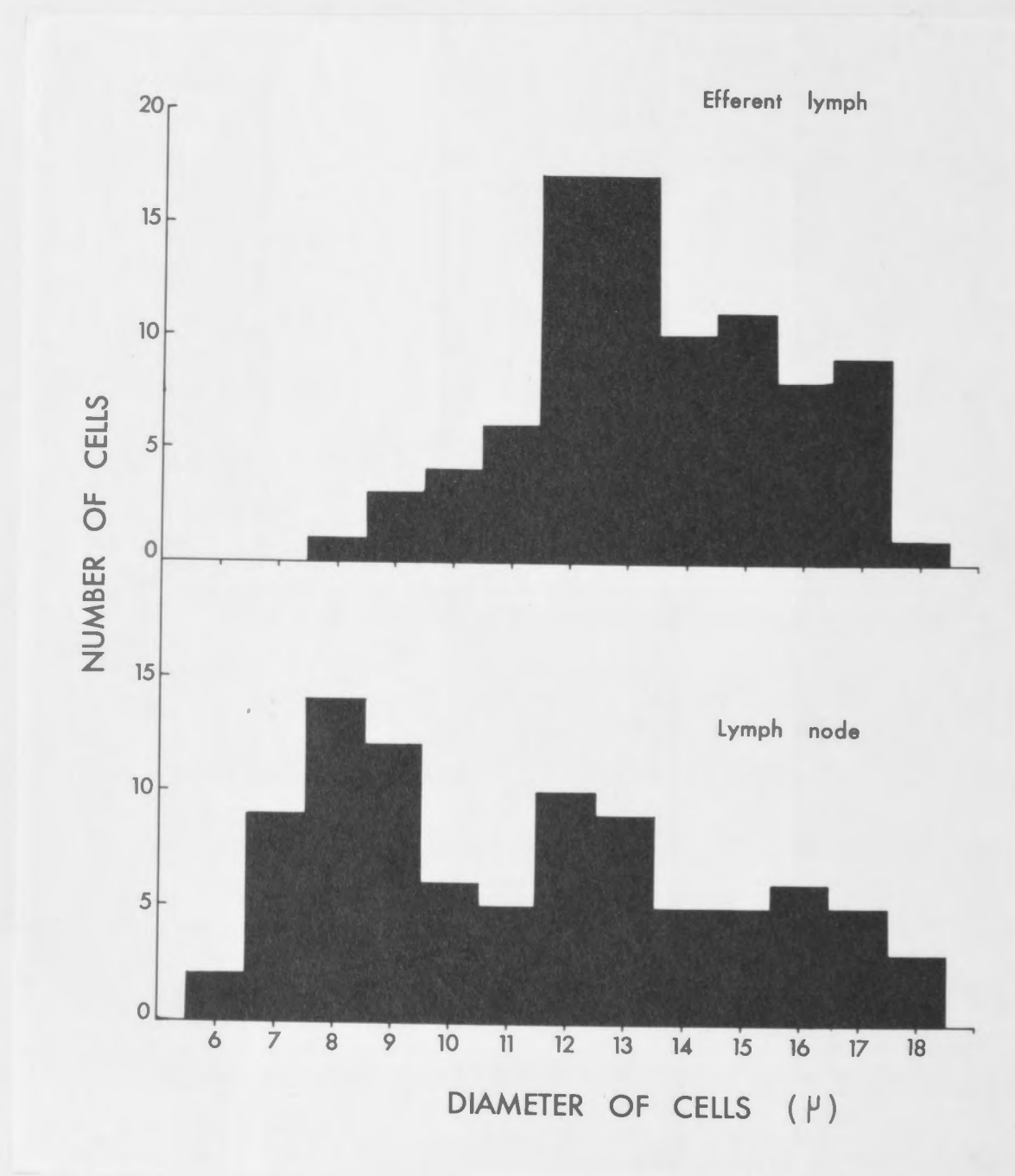


Fig. 4.5. Frequency distribution of the diameters of antibody-forming cells from the efferent lymph and the lymph node.

### C. THE MORPHOLOGY OF ANTIBODY-PRODUCING CELLS.

#### I. Light microscopy.

87 undamaged cells from lymph and 91 from lymph nodes were examined at various times during the primary and secondary responses. There was no consistent morphological difference between cells showing plaque formation, or agglutination and adherence of erythrocytes, or between antibody-forming cells from primary or secondary responses. (fig. 4.6).

The histograms in fig. 4.5 show the frequency with which antibody-forming cells of different average diameters were found. Two groups of active cells could be distinguished. The cells of the first group were large, usually from 12-17 $\mu$  in diameter with a nucleus occupying most of the apparent area of the smeared cell. These cells showed varying degrees of basophilia, although about 10% of them, all with diameters in the range 15-17 $\mu$ , were pale-staining blast-like cells. Nearly all the antibody-forming cells in the efferent lymph, and about half of those obtained from the node, belonged to this group.

The second group of active cells were commonly found only in the lymph nodes. These cells were 6-10 $\mu$  in diameter, intensely basophilic, and had a relatively small nucleus. They correspond to the

Fig. 4.6. Antibody-producing cells (Leishman's stain).  
(a)-(h), efferent lymph cells, (i)-(l), lymph node cells.  
Cell (i) is releasing while in mitosis. Cells (j), (k),  
(l), are mature plasma cells, found only in the node.

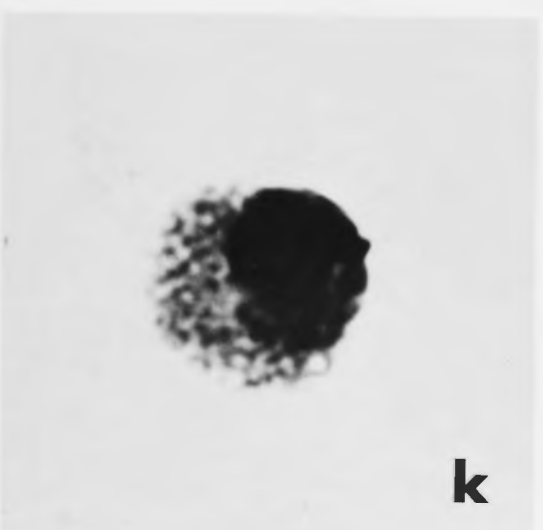
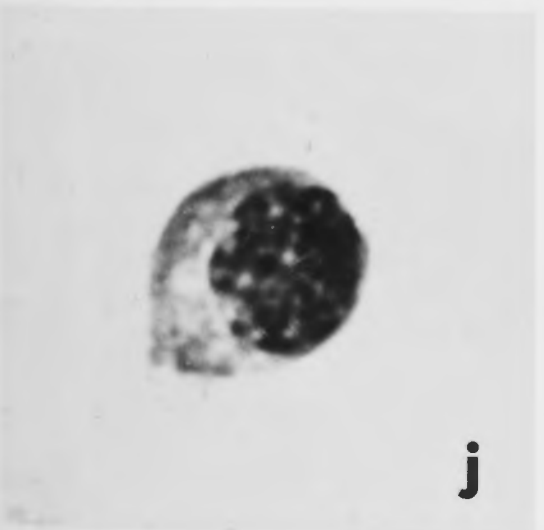
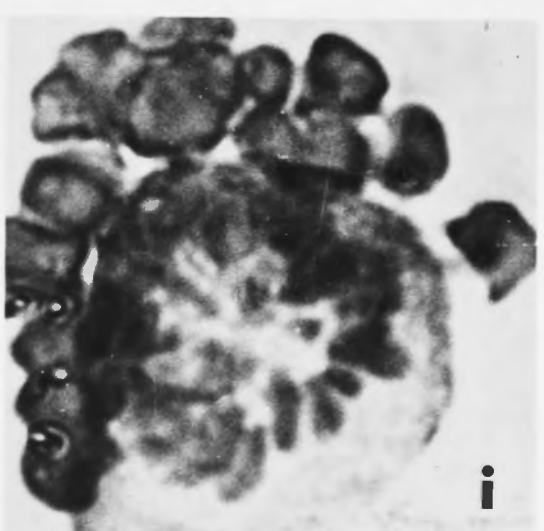
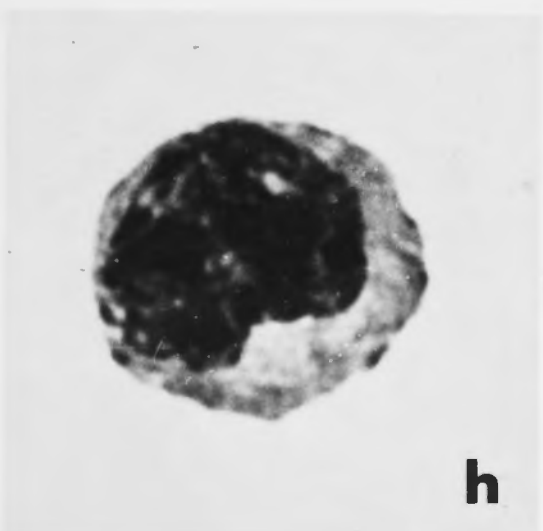
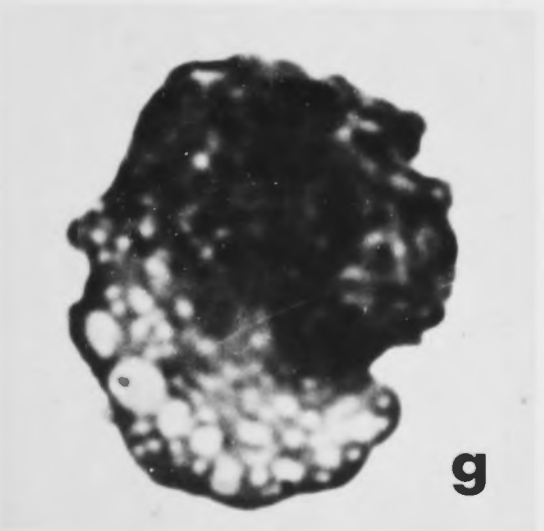
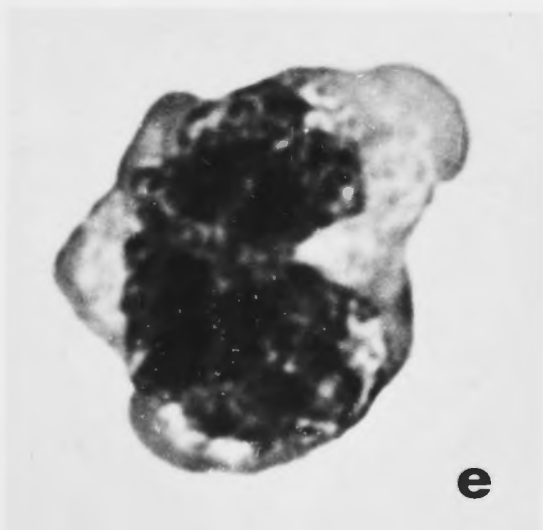
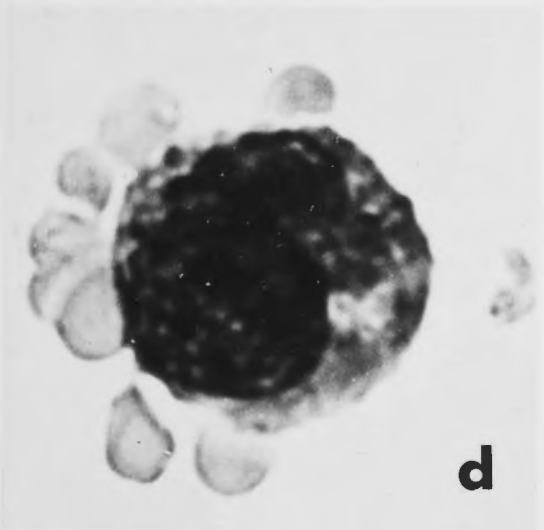
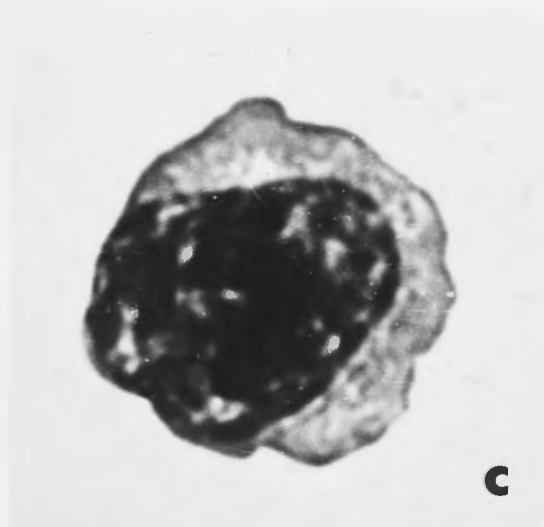
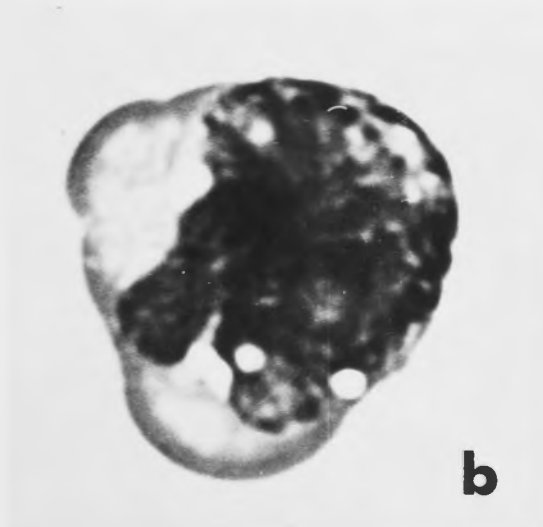
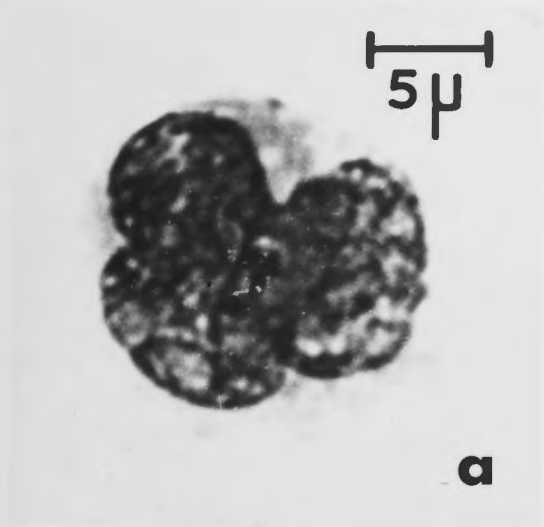




Fig. 4.7. Antibody-forming cell from efferent lymph.  
ER = endoplasmic reticulum; N = nucleus; M = mitochondria; E = sensitised erythrocyte. The cytoplasm contains numerous free ribosomes, either single or in small clusters. x27,000

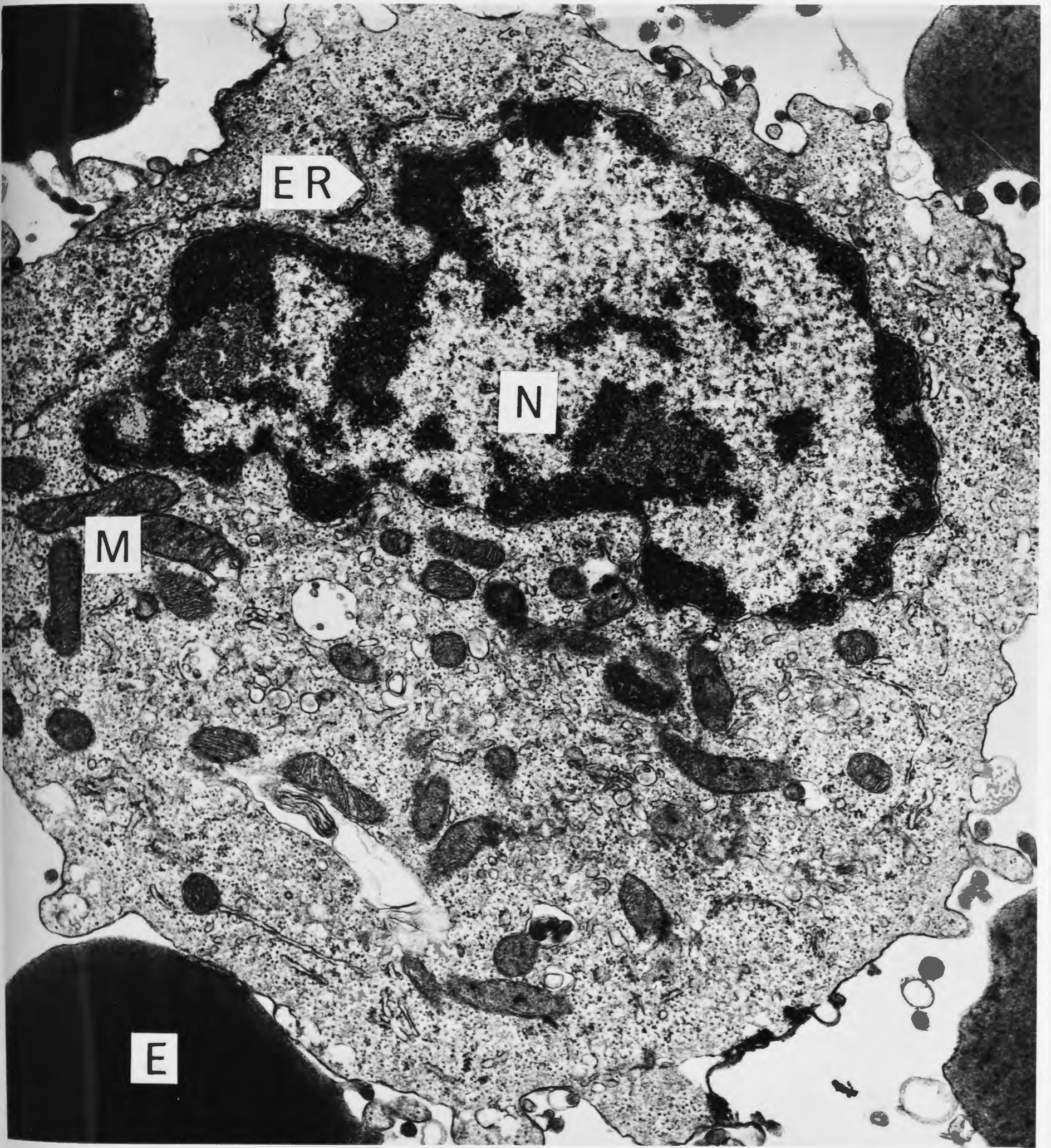


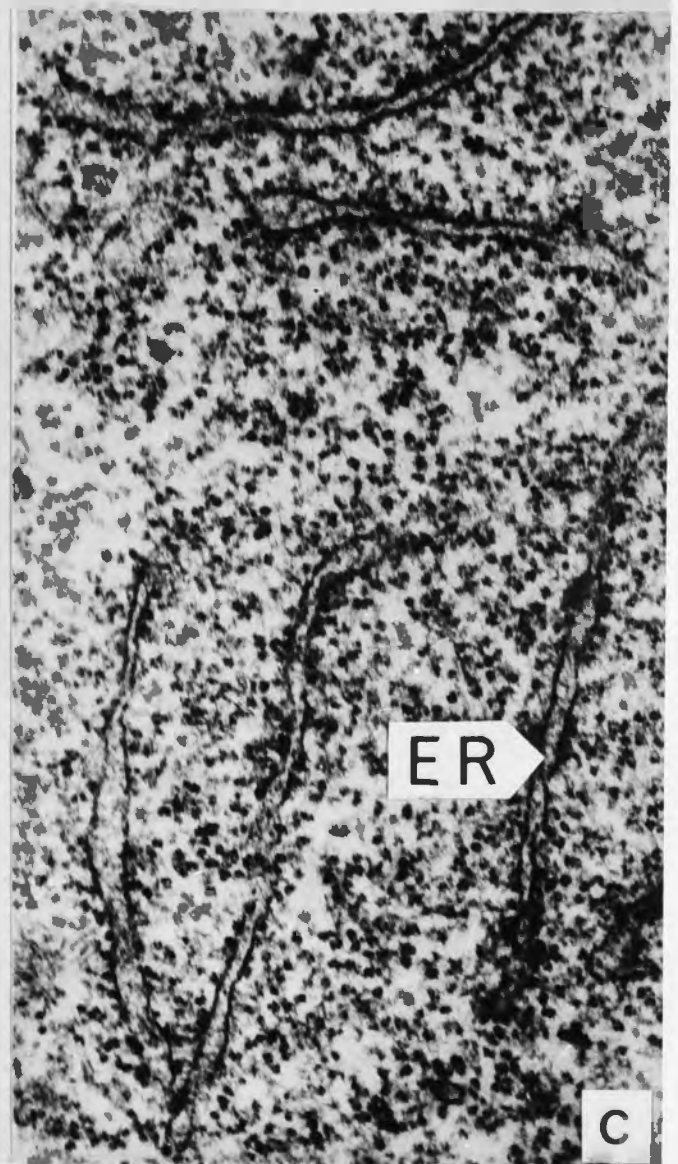
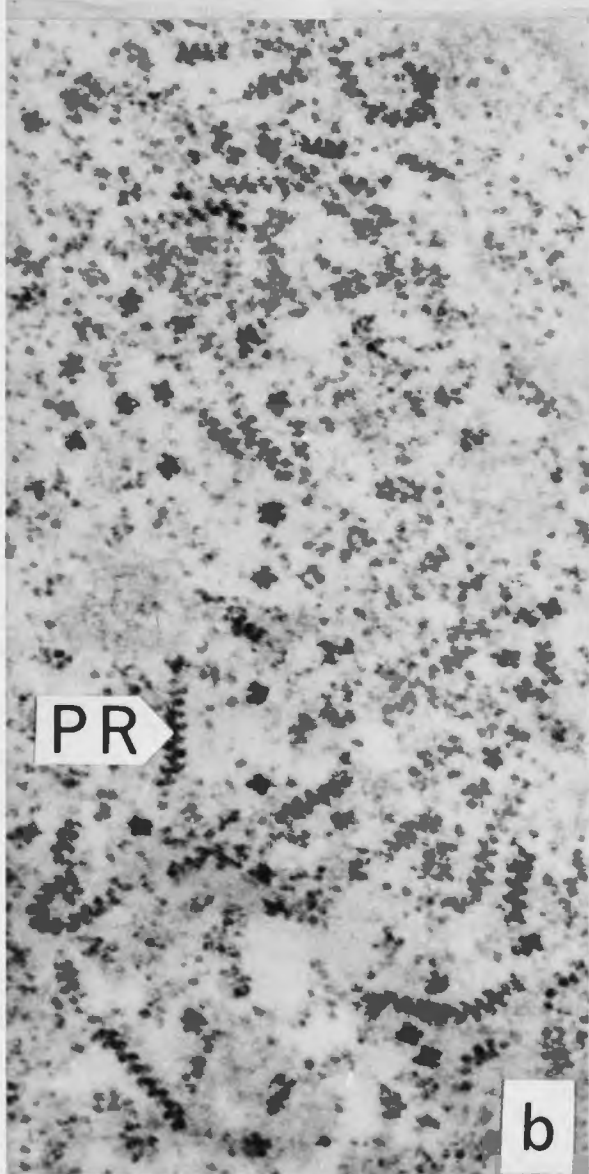
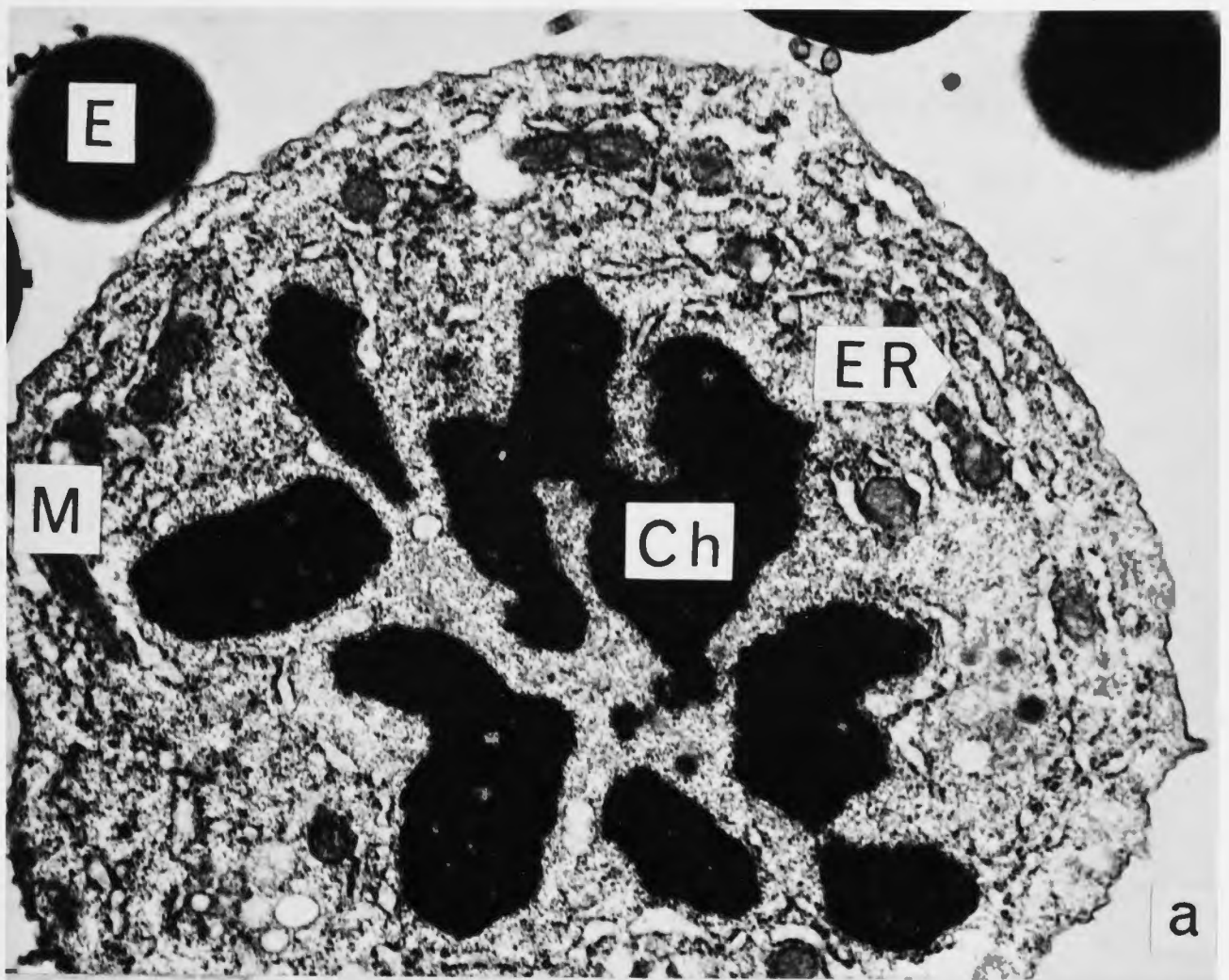
Fig. 4.8.

(a). Antibody-producing cell in mitosis, from the lymph node. ER = Well-developed endoplasmic reticulum; M = mitochondria; E = sensitised erythrocyte; Ch = chromosome.  $\times 12,000$

(b). Helical polyribosomes (PR) in the cytoplasm of an antibody-forming cell from efferent lymph.  $\times 45,000$

(c). Endoplasmic reticulum (ER) in the cytoplasm of an active cell from the lymph node.  $\times 45,000$







mature plasma cells of many authors. Cells intermediate between the two types were found. An area of clear cytoplasm near the nucleus was common in the plasma cells, and was sometimes seen in the large basophilic cells. Cytoplasmic inclusions which may have been Russel bodies were occasionally found (fig. 4.6).

## II. Electron microscopy.

Ten clumps containing a total of 305 cells were prepared for electron microscopy. About 20% of these cells were examined in section. Cells from efferent lymph or lymph nodes were clumped separately.

The efferent lymph cells have large nuclei, commonly exhibiting a marked indentation of one side, with dense clumps of chromatin often packed against the nuclear membrane (fig. 4.7). The cytoplasm was densely packed with ribosomes, many of which were in the form of clusters. A few flattened sacs of rough endoplasmic reticulum were seen. Mitochondria of various sizes were plentiful and the Golgi apparatus was well developed. Centrioles and associated structures (satellite bodies and microtubules) were often seen. Large polyribosomes, estimated to contain up to 50 or more ribosomes apparently arranged in a helix, were sometimes seen. In one cell

(fig. 4.8b) these were abundant throughout the cytoplasm. These cells correspond to the large basophilic mononuclear cells seen by light microscopy.

Antibody-producing cells in the node were of 2 types, corresponding to the 2 groups distinguished by light microscopy. Many resembled the cells found in efferent lymph. A minority, the plasma cells found only in the node, were small with considerable amounts of rough endoplasmic reticulum in the cytoplasm (fig. 4.8a). Again cells morphologically intermediate between the 2 types could be found.

in the lymph may be involved (table 4.3). The other basophilic cells may well be producing antibody to other antigens from the bacteria. In the node itself, the proportion of active cells is approximately one-tenth that of the lymph at the peak of the response, (fig. 4.4), but numbers of antibody-forming cells decline more slowly than in the lymph.

Preliminary observations on popliteal afferent lymph indicate that it does not carry antibody-formers, although the lymph flowing from a subcutaneous granuloma towards the popliteal node contains very large basophilic mononuclear cells similar in appearance to the active cells of the node and efferent lymph. The possible specialized role of lymph nodes in the

#### D. DISCUSSION.

The popliteal node of the sheep responds to stimulation with boiled Salmonella by releasing about  $5 \times 10^7$  detectable antibody-producing cells, mostly from 2-6 days after the stimulus. This represents roughly 0.5% of the total cell output during this time. At the peak of the response, from 1 in 100 to 1 in 20 of the efferent lymph cells are releasing specific antibody directed against the bacterial lipopolysaccharides and as many as 1 in every 2 basophilic cells in the lymph may be involved (table 4.3). The other basophilic cells may well be producing antibody to other antigens from the bacteria. In the node itself, the proportion of active cells is approximately one-tenth that of the lymph at the peak of the response, (fig. 4.4), but numbers of antibody-forming cells decline more slowly than in the lymph.

Preliminary observations on popliteal afferent lymph indicate that it does not carry antibody-formers, although the lymph flowing from a subcutaneous granuloma towards the popliteal node contains many large basophilic mononuclear cells similar in appearance to the active cells of the node and efferent lymph. The possible specialized role of lymph nodes in the

generation of antibody-forming cells could be conveniently studied in sheep with the efferent and one afferent duct cannulated on either side of the same node.

Some of the problems encountered by Dent and Good in using the Jerne and the Zaalberg techniques to assay haemolysin and haemagglutinin-producing cells in the fowl, have been recently discussed (Dent and Good, 1965). The free suspension technique used here increases the sensitivity of plaque detection, and allows adherence-producing cells to be assayed at the same time, without any danger of non-specific clumping of lymphoid cells and erythrocytes. Evidence has been presented that plaque formation, immuno-cyto-adherence and localised agglutination are due to antibody release by the central lymphoid cell. It seems almost certain that these cells are synthesizing the antibody which they release although the evidence is only circumstantial. All the active cells show differentiation for protein synthesis and limited secretion; varying degrees of basophilia by light microscopy, large numbers of ribosomes and some endoplasmic reticulum by electron microscopy. The fact that puromycin reversibly inhibits plaque formation and agglutination is not conclusive proof



that the active cells are actually synthesizing antibody since a mechanism which depends on protein synthesis may be required to transport stored antibody across the plasma membrane. The cumbersome alternative explanation to antibody synthesis however, is that the basophilic cells store antibody which they receive from other cells.

Most of the plaques produced by the sheep cells in this system are very small (fig. 4.1a). The size of a plaque presumably depends on the number of antibody molecules produced by a cell, and on their lytic efficiency. So a small plaque would be expected if the central cell released small amounts of antibody or if a lot of antibody molecules were required to lyse one sensitised erythrocyte. These alternatives cannot be distinguished in this system. However, as in other animals (Baney et al. 1962; Lo Spalluto et al. 1962; Weidanz et al. 1964), virtually all the antibody produced by the sheep in response to Salmonella antigens was 19S, and macroglobulins are believed to be extremely efficient at binding complement and lysing red cells, at least in the rabbit anti-sheep red cell system (Humphrey and Downmashkin, 1965; Borsos and Rapp 1965). If this is also true of sheep 19S antibody reacting against lipopolysaccharide-coated erythrocytes, then

it is possible that the majority of efferent lymph cells were releasing very small amounts of antibody in the test chambers. Mouse lymph node cells releasing antibacterial antibody in similar preparations produce a majority of plaques approximately 400 $\mu$  in diameter with a few ranging in size down to the limit of sensitivity of this technique, the lysis of 10-20 red cells (Chapter 2, fig. 2.1).

Agglutination and adherence are a less convenient index of antibody production than plaque formation because relatively few cells may be examined for these effects. One plaque-forming cell amongst  $10^6$  inactive cells can be immediately seen when chambers are inspected under low power (X60), but only  $10^4$  cells can be conveniently scanned under higher power (X240) for adherence of erythrocytes. Even in the presence of complement, many active lymphoid cells are unable to lyse attached or locally agglutinated erythrocytes. The antibody which these cells produce is probably qualitatively different from the antibody released by plaque-forming cells. It exhibits the fundamental property of binding to its corresponding antigen, but is unable to bring about lysis.

Localised agglutination seems to differ from immuno-cyto-adherence only in the amount of antibody

produced by the active cell. Agglutination obviously involves the release of free antibody into the medium, and occurs at  $37^{\circ}\text{C}$  but not at  $0^{\circ}\text{C}$ . Cells showing immuno-cyto-adherence on the other hand, apparently have antibody attached to their surface, either because this antibody is in the process of escaping from the cell or because release has stopped. The inhibition of adherence caused by prewashing cells with free homologous lipopolysaccharide at  $0^{\circ}\text{C}$  can be quickly reversed by raising the temperature to  $37^{\circ}\text{C}$ ; this suggests that the antibody responsible for binding erythrocytes to the lymphoid cell is being continuously released during incubation. Exactly how antibody attaches an erythrocyte to a lymph cell is obscure; it may be that the sensitised erythrocyte is bound by several molecules at once, which are continually renewed as more antibody is formed. The bond is quite firm and often remains intact during the smearing and drying of the cells (figs. 4.6d, 4.6i).

The functional criteria established for the identification of single antibody-forming cells in this system were used to isolate single cells for morphological studies. Most of the active cells examined by light microscopy resembled the blast cells and

immature and mature plasma cells of Nossal (1959) and other workers. However, the cells studied were a rather restricted population; they were all isolated from lymphoid tissue at or near the peak of primary and secondary responses, and were all probably releasing 19S antibody. A much greater variety of morphological types could be found in mice when cells releasing 19S or 7S antibody were studied throughout the entire immune response to sheep erythrocytes (chapter 5).

Very recently, Harris and co-workers (Harris et al. 1966; Hummeler et al. 1966), have described the electron microscope appearance of haemolysin-producing cells from rabbit lymph and lymph nodes. Their results are in full agreement with those reported here, except that most of the cells they studied were very much smaller than the corresponding sheep cells; this may reflect a real difference between the species, or a difference in techniques used to examine the cells. In the rabbit as in the sheep, the antibody-forming cells of efferent lymph contained numerous ribosomes but very little organised endoplasmic reticulum. These cells may be secreting very small amounts of actual protein in the form of antibody molecules of high activity, or they may be using some unrecognised means of transporting larger amounts out of the cell.



Protein secretion is usually associated with considerable amounts of rough endoplasmic reticulum. (Kurosumi, 1961). Active plasma cells with this type of cytoplasmic organisation were identified, in agreement with recent reports that such cells are capable of releasing haemolytic antibody. (Harris et al. 1966; Binet and Bussard, 1964; Fitch et al. 1965). These plasma cells were found only in the lymph nodes.

The large basophilic mononuclear cells contain many free ribosomes and polyribosomes, structures which have long been known to be associated with rapid growth and differentiation (Palade, 1955). Large polyribosomes of apparently helical form were sometimes found, similar to those previously described in a number of differentiating tissues (Behnke, 1963; Waddington and Perry, 1963; Echlin, 1965). In most cells, one or two helical polyribosomes could be found per section. In one cell the cytoplasm was packed with them (fig. 4.8b); conceivably all the large basophilic cells could pass through a developmental stage in which they contain many of these structures.

No conclusions on the developmental history of antibody-forming cells in sheep lymphatics can be drawn from the observations made in this study. However, Hall et al. (1967), have shown that the cells of the

popliteal efferent lymph play an important part in the immune response of the sheep; serum antibody titres are drastically reduced if the efferent lymph cells collected from a stimulated node are not returned to the animal. These cells can also initiate an antibody response in a chimaeric twin which has had no experience of the antigen. This and the fact that plasma cells occur in the node and not in the lymph, supports the idea (Hall et al. 1967) that many of the basophilic cells of efferent lymph settle in nodes further along the lymphatic chain and develop there into antibody-producing plasma cells.

For light microscope examination, individual antibody-forming cells were secured in serum and stained with Leishman's stain. For electron microscopy, a number of active cells were clumped with antiserum to form a specimen of convenient size, then sectioned. Most of the active cells from efferent lymph are large and basophilic, while a small proportion are blast-like. These cells contain abundant free ribosomes and very little endoplasmic reticulum.

E. SUMMARY.

The antibody-forming cells which appear in the popliteal lymph node and efferent lymph of the sheep following immunization with boiled Salmonella have been studied by light and electron microscopy. Cells were incubated in monolayers with target erythrocytes sensitised with bacterial lipopolysaccharide. Three types of interaction between a proportion of the lymph cells and the erythrocytes surrounding them have been shown to indicate antibody formation; plaque-formation, immuno-cyto-adherence, and localised agglutination.

At the peak of the response, 4 days after antigenic stimulation approximately 1 cell in every 200 from lymph node suspensions produces detectable specific antibody, while up to 1 cell in 20 in the lymph is active.

For light microscope examination, individual antibody-forming cells were smeared in serum and stained with Leishman's stain. For electron microscopy, a number of active cells were clumped with antiserum to form a specimen of convenient size, then sectioned. Most of the active cells from efferent lymph are large and basophilic, while a small proportion are blast-like. These cells contain abundant free ribosomes and very little endoplasmic reticulum.

In the node only, an additional class of antibody-forming plasma cells are found which have considerable amounts of endoplasmic reticulum in their cytoplasm.

CHAPTER 5.

THE CELLULAR RESPONSE TO ANTIGENIC STIMULATION

IN THE MOUSE.



In the previous chapter, a study was made of sheep lymphoid cells producing macroglobulin antibody. To compare cells releasing 19S and 7S antibody, a different system was required. This chapter describes the response of the popliteal lymph nodes of mice to stimulation with sheep erythrocytes. Detailed curves of the cellular response with time after primary and secondary immunization have been constructed, and single antibody-forming cells have been examined by light microscopy throughout these responses.

## CHAPTER 5.

### THE CELLULAR RESPONSE TO ANTIGENIC STIMULATION IN THE MOUSE.

#### I. Introduction.

Ultracentrifugation distinguishes 2 groups of haemolysin in the mouse (Miller, 1965): high molecular weight antibody with a sedimentation constant of around 19S, and lower molecular weight antibody with a sedimentation constant of 7S. These 2 groups seem to differ greatly in their haemolytic efficiency (Miller and Wigzell, 1965). It is found that as little as 1 molecule of macroglobulin (19S) haemolysin may lyse a sheep red cell in the presence of complement, whereas an average of several hundred molecules of 7S antibody

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A. DIFFERENTIAL DETECTION OF CELLS RELEASING  
ANTIBODY OF HIGH OR LOW HAEMOLYTIC EFFICIENCY.

I. Introduction.

Ultracentrifugation distinguishes 2 groups of haemolysin in the mouse (Adler, 1965); high molecular weight antibody with a sedimentation constant of around 19S, and lower molecular weight antibody with a sedimentation constant of 7S. These 2 groups seem to differ greatly in their haemolytic efficiency (Möller and Wigzell, 1965). It is thought that as little as 1 molecule of macroglobulin (19S) haemolysin may lyse a sheep red cell in the presence of complement, whereas an average of several hundred molecules of 7S antibody

with 1 much non-lytic molecule attached to a red cell,

is required (Humphrey and Dourmashkin, 1965; Borsos and Rapp, 1965). It may be that 2 molecules of the smaller 7S antibody must attach close together on the surface of the target cell in order to bind complement; a relatively high concentration of 7S antibody would be needed for such a close attachment to be a common event.

The original Jerne-Ingraham plaque technique probably detects only cells releasing 19S haemolysin; the close correspondence between plaque-forming cells and serum titres of macroglobulin antibody has been described by several authors (Möller and Wigzell, 1965; Jerne et al. 1963). The later 7S response coincides with decreasing numbers of plaque-forming cells.

Recently, Sterzl and Riha (1965) and Dresser and Wortis (1965) have extended the plaque technique to assay cells releasing antibody of low haemolytic efficiency. Immunized mouse lymphoid cells are incubated with target erythrocytes as in the Jerne technique. Antibody of low lytic power diffuses from a proportion of these cells and attaches to the surrounding erythrocytes but does not lyse them. Now addition of antiserum directed against mouse gamma-globulin will "develop" these plaques. It is thought that when a number of antiglobulin molecules combines with 1 such non-lytic molecule attached to a red cell,

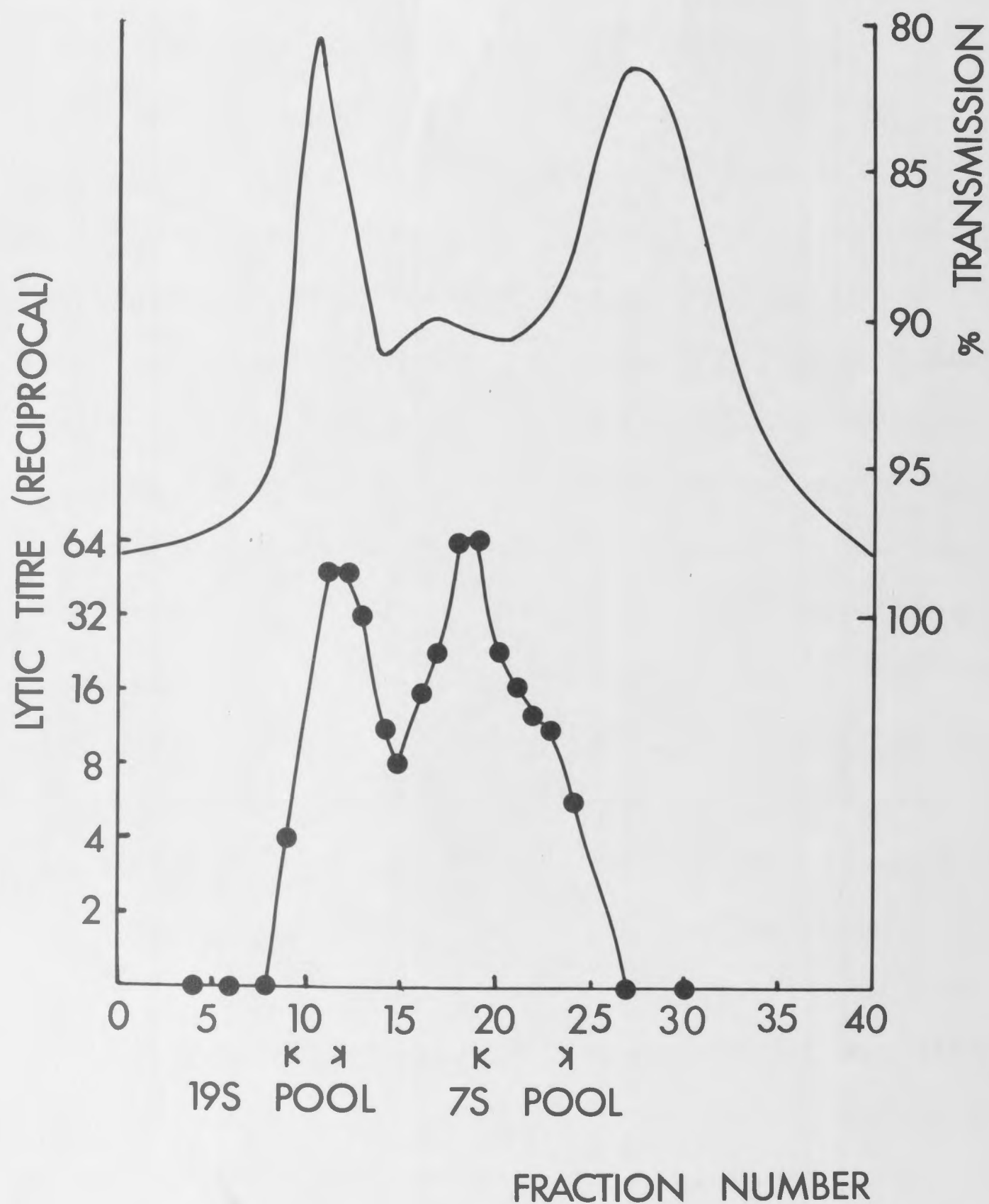


Fig. 5.1.

Preparation of 19S and 7S mouse haemolysin by filtration through "sephadex G-200". Black circles connect lytic titres of the fractions collected. The upper line represents the percentage transmission traced by an LKB Produkter AB recorder scanning at a wavelength of approximately 280mμ.



the resulting complex is able to bind complement and so lyse the cell. (Dresser and Wortis, 1965).

## II. Standardizing the antiglobulin technique: Serum.

Gamma-globulin was obtained from mouse serum by sodium sulphate precipitation (Kekwick, 1940). Protein with the mobility of gamma-globulin was the only constituent of this preparation detectable by cellulose acetate electrophoresis. Approximately 5mg of this gamma-globulin in Freund's adjuvant was injected subcutaneously into each of 2 rabbits. The rabbits were bled 11 and 22 days later. On day 22, they were reinjected intravenously with gamma-globulin, and 7 days after this booster, bled a third time. Thus 6 antiglobulin sera were obtained.

The antiglobulin sera were tested for their ability to enhance the titre of 19S and 7S mouse haemolysin. A pool of serum from 10 mice hyperimmunized with sheep red cells was filtered through a column of "Sephadex G-200" gel, to provide 19S and 7S fractions (fig. 5.1). Seven replicate 2-fold dilution series of 7S mouse haemolysin were made in a perspex tray (mechanics of the test as described in appendix 2.2). 0.025ml of 5% sheep red cells was added to each well, and the tray was incubated for 1 hour at 37°C.

Table 5.1

Enhancement of the lytic effect of mouse haemolysin by rabbit anti-mouse-gamma globulin. Figures represent estimated extent of lysis of sheep red cells. "4" means 100% lysis, "0" means no lysis.

7S mouse haemolysin (reciprocal of dilution).

		16	32	64	128	256	512	1024	2048	4096	8192
Rabbit	20	0	0	0	1	2	2	2	2	2	0
antiglobulin	40	0	1	2	2	2	3	3	2	1	0
(reciprocal	80	1	2	3	4	4	4	4	3	2	1
of	160	2	3	4	4	4	4	4	3	3	1
dilution).	320	2	3	3	4	4	4	3	3	2	1
	640	3	2	2	3	3	3	3	2	2	1
	1280	4	2	2	2	2	2	1	1	1	0

19S mouse haemolysin (reciprocal of dilution).

		16	32	64	128	256	512	1024	2048	4096	8192
Rabbit	20	0	0	0	0	0	0	0	0	0	0
antiglobulin	40	0	0	0	0	0	0	0	0	0	0
(reciprocal	80	0	0	0	0	0	0	0	0	0	0
of	160	0	0	0	0	0	0	0	0	0	0
dilution).	320	2	1	0	0	0	0	0	0	0	0
	640	4	3	2	0	0	0	0	0	0	0
	1280	4	4	3	2	0	0	0	0	0	0

Dilutions of one rabbit anti-mouse-gamma-globulin serum, previously absorbed with sheep red cells, were added to the tray to provide a checkerboard, as shown in table 5.1. After a further 15 minutes' incubation, complement was added, and the tray reincubated for 45 minutes at 37°C, then read for lysis of erythrocytes. A similar test was performed on the 19S mouse haemolysin pool.

In table 5.1. contour lines have been drawn to connect wells showing 75% lysis. In the lower left-hand corner of both trays the effect of the 7S or 19S mouse antibody alone can be seen; higher concentrations of rabbit antiglobulin inhibited the lytic action of 19S antibody. From the 7S tray it is evident that the enhancing effect of the antiglobulin was concentration dependent; very high concentrations inhibited lysis of erythrocytes by 7S mouse antibody. A dilution of about 1/160 was optimal, and this enhanced the lytic titre approximately 200-fold. By contrast, no enhancement of 19S mouse antibody was detected.

Table 5.2 compares the enhancing effect of all 6 rabbit antiglobulin antisera on lysis of sheep erythrocytes by 19S or 7S mouse haemolysin. There is considerable variation between antiglobulin sera in extent to which 7S lysis was enhanced. None of the

TABLE 5.2

Comparison of the enhancing effect of 6 antiglobulin antisera on lytic titre of mouse 7S and 19S haemolysin.

Rabbit. no.	Antiserum	Final dilution of antiglobulin	<u>7S haemolysin.</u>
			Estimated 50% lysis end point.
-	control, no serum		30
1	11 day primary	1/50	1500
1	22 day primary	1/100	5000
1	7 day secondary	1/100	15000
2	11 day primary	1/50	1500
2	22 day primary	1/100	750
2	7 day secondary	1/100	15000
			<u>19S haemolysin.</u>
-	control, no serum		100
1	11 day primary	1/50	100
1	22 day primary	1/100	100
1	7 day secondary	1/100	50
2	11 day primary	1/50	100
2	22 day primary	1/100	100
2	7 day secondary	1/100	30



antisera enhanced lysis by 19S antibody. Two antisera slightly depressed the titre of 19S haemolysin.

### III. Standardizing the antiglobulin technique: Cells.

The appearance of a class of cells which produce plaques only in the presence of antiglobulin correlates well with the rise of serum 7S lytic antibody titres as described by Adler (see below). Such cells will be referred to in this chapter as producers of 7S antibody. Possible objections to this interpretation are raised in the "Discussion". Two types of preparation have been used in this study to assay mixed populations of 19S- and 7S- antibody-forming cells; Jerne plates, and microdrops under oil.

#### (a). Agar plates.

Plates were prepared essentially as described by Jerne et al. (1963), except that DEAE-dextran was incorporated in the overlay agar at a concentration of 8mg/ml.

In analysing populations containing both types of antibody-forming cell, it would obviously be most useful to perform sequential assays on the same sample. If the 19S plaques could be counted and marked on an agar plate, as suggested by Sterzl and Riha (1965), a very small number of additional 7S plaques could

TABLE 5.3

The effect of concentration of antiglobulin on the development of 7S plaques; and a comparison of the number of such plaques detected when complement was added before or after antiglobulin.

Number of plaques counted.			
		Group A. Complement added before antiglobulin	Group B. Complement added after antiglobulin
	50	74	108
Dilution	100	60	146
of	200	50	155
antiglob.	400	48	112
(recip).	800	23	67
	1600	15	46

then be detected after the addition of antiglobulin. By contrast, a minority population of 7S-producing cells might not be recognized if 2 plates were assayed in parallel, one with antiglobulin and one without; Poisson variation between the plates would obscure small but real differences. However, a complication arose when it was found that complement and rabbit antiglobulin "competed" with one another in Jerne-type preparations; addition of complement before antiglobulin decreased the number of 7S plaque detected, while antiglobulin interfered with the development of 19S plaques by complement.

Table 5.3 demonstrates interference by complement with the appearance of 7S plaques. Lymph nodes were obtained from a hyperimmunized mouse in which nearly all the active cells were producing 7S antibody. Each of 12 replicate samples from this population was incorporated into 1ml of "overlay mixture" containing 0.7% agar and 2% erythrocytes in Eagle's medium, and these samples were poured on to 12 plates of basal agar. All plates were incubated at 37°C throughout the experiment. To each of 6 of the plates (group A in table 5.3), 1ml of 1/10 complement was added after 1 hour's incubation. This was washed off with Eagle's medium 30 minutes later, and replaced by 1ml of the

TABLE 5.4

Interference by antiglobulin with the development of plaques formed by 19S antibody.

Plate	Antiglobulin before or after complement	Counts	Total
1	before	62	148
2	before	86	
3	after	114	215
4	after	101	



stated dilution of antiglobulin. This was in turn washed off after a further hour, and a second sample of complement was pipetted on to the plates for the last 30 minutes, before counting. In group B, antiglobulin was added after  $1\frac{1}{2}$  hours initial incubation, then replaced by complement at  $2\frac{1}{2}$  hours for a further 30 minutes, before plaques were counted. Repeated experiments of this type have shown that sequential assay of 19S and 7S plaque-producers on the same plate results in about 50% underestimation of the number of cells releasing 7S antibody. Table 5.3 also demonstrates that 7S plaques fall off in number as the concentration of antiglobulin decreases, in much the same way as does the titre of 7S mouse antiserum.

Table 5.4 shows the reverse type of interference effect; a significant decrease in the number of plaques detected in a pure 19S population when antiglobulin was added before complement. Four replicate plates were incubated for 1 hour at  $37^{\circ}\text{C}$ . Antiglobulin was then added to numbers 1 and 2, Eagle's medium to 3 and 4, and all were reincubated for another hour. The antiglobulin or Eagle's was then poured off and replaced by complement for a final 30 minutes of incubation. Antiglobulin suppressed numbers of 19S

plaques by about 30%.

Separate series of plates were used to detect only cells producing 19S antibody on the one hand, or both 19S and 7S-releasing cells on the other. An estimate of 7S producers was obtained by subtracting 50% of the number of 19S plaques from the numbers counted on the plates to which antiglobulin had been added. Jerne plates were usually incubated for 3 hours. Incubation for 24 hours increased numbers of 19S and 7S plaques by about 25%, without curing interference effects.

(b). Microdrops.

Stable monolayers of lymphoid cells and target erythrocytes may be incubated in droplets of Eagle's medium under oil, as described in chapter 3. This technique detects plaques with the same sensitivity as the standard free suspension assay of cells in sealed chambers. It was found that sequential assays of 19S and 7S-producing cells could be performed if these monolayers in droplets were incubated for 30 minutes with complement to develop 19S plaques, then anti-globulin at a dilution of 1/50 was instilled carefully into the top of each drop over the monolayer. 7S plaques were scored after a further 15-30 minutes incubation. No problems of interference between complement and antiglobulin were encountered in this

system, although 19S plaques were considerably decreased in size if exposed to antiglobulin before complement, and vice versa for 7S plaques.

(c). Summarizing methods used for detection of both 19S and 7S antibody cells from mouse lymph nodes.

Sequential type assays for the 2 classes were performed in microdrops whenever the numbers of active cells in the nodes were relatively high. Use of this method allowed detection of small numbers of 7S producers in presence of larger numbers of 19S plaques. The sequential assay was thus especially valuable at the time when a switchover of 19S to 7S production was occurring. However, it was not practicable to plate out more than 1 or 2 million cells by this method.

At the extremes of the immune response, parallel assays were done, of necessity, in Jerne plates. Lymph nodes were broken up by gentle rubbing on wire mesh (appendix 2.1) and fragments of stroma as well as cells were plated.

TABLE 5.5

Response of spleens and popliteal lymph nodes to injection of various numbers of sheep erythrocytes.

Group 1. Intravenous injection.

Mouse no.	No. of eryths. injected	Plaque-forming cells/ $10^6$ in spleen
1	$10^9$	1210
2	$10^9$	2540
3	$10^8$	2680
4	$10^8$	570
5	$10^7$	2540
6	$10^7$	500
7	$10^6$	< 50
8	$10^6$	40
9	$10^5$	< 16
10	$10^5$	< 16

Group 2. Injection into both footpads.

Mouse no.	No. of eryths injected	Plaque-forming cells/ $10^6$ .		
		Left node	Right node	Spleen
1	$2 \times 10^8$	453	510	540
2	$2 \times 10^8$	625	395	93
3	$10^7$	1060	660	46
4	$10^7$	1180	1040	97
5	$10^6$	< 16	< 13	13
6	$10^6$	50	420	10
7	$10^5$	< 16	< 7	< 5
8	$10^5$	< 50	< 15	< 5



B. RESPONSE OF MOUSE LYMPH NODES AND SPLEENS TO VARIOUS  
DOSES OF SHEEP ERYTHROCYTES.

This experiment was performed to define a suitable dose of sheep red cells for use in all subsequent immunizations of mice. The dose-response relationship has been determined for the primary response only.

Ten mice were injected intravenously in the lateral tail vein with various numbers of sheep erythrocytes, and the spleens assayed for 19S antibody-forming cells 4 days later. A further group of 8 mice were injected in both footpads with sheep erythrocytes at different dilutions, and both popliteal lymph nodes and spleens assayed 5 days later. Results are shown in table 5.5.

For both lymph nodes and spleen, a dose of  $10^7$  or more sheep erythrocytes seemed to elicit a maximal response, while there was a sharp drop in the response to smaller amounts of the antigen. Injection of  $2 \times 10^8$  sheep red cells into the footpads of 1 mouse (number 1, group 2) gave rise to a vigorous response in the spleen, probably because sufficient antigen ( $10^7$  red cells?) reached the blood stream.

TABLE 5.6

Absence of antigenic cross-stimulation between opposite popliteal lymph nodes.

Mouse group no.	Time after secondary stimulation (days).	Plaque-forming cells/ $10^6$	
		left nodes	right nodes
1	2	380	17
2	3	500	17
3	4	50	<10

C. INDEPENDENT RESPONSE OF OPPOSITE LYMPH NODES.

One reason for studying the cellular response of mouse popliteal lymph nodes rather than spleens was that twice as many organs could be examined in the same number of inbred animals. The following test showed that very little cross-over of antigen occurred between the 2 nodes.

Six mice were injected in each footpad with  $10^8$  sheep red cells. One month later, the left footpads only of all 6 were reinjected. Mice were killed in groups of 2 at 2, 3, and 4 days after this secondary antigenic stimulation, and the 19S plaque-forming cells in left and right nodes compared.

Table 5.6 shows that if antigen reached the nodes on the uninjected sides, it was in amounts too small to cause secondary stimulation.

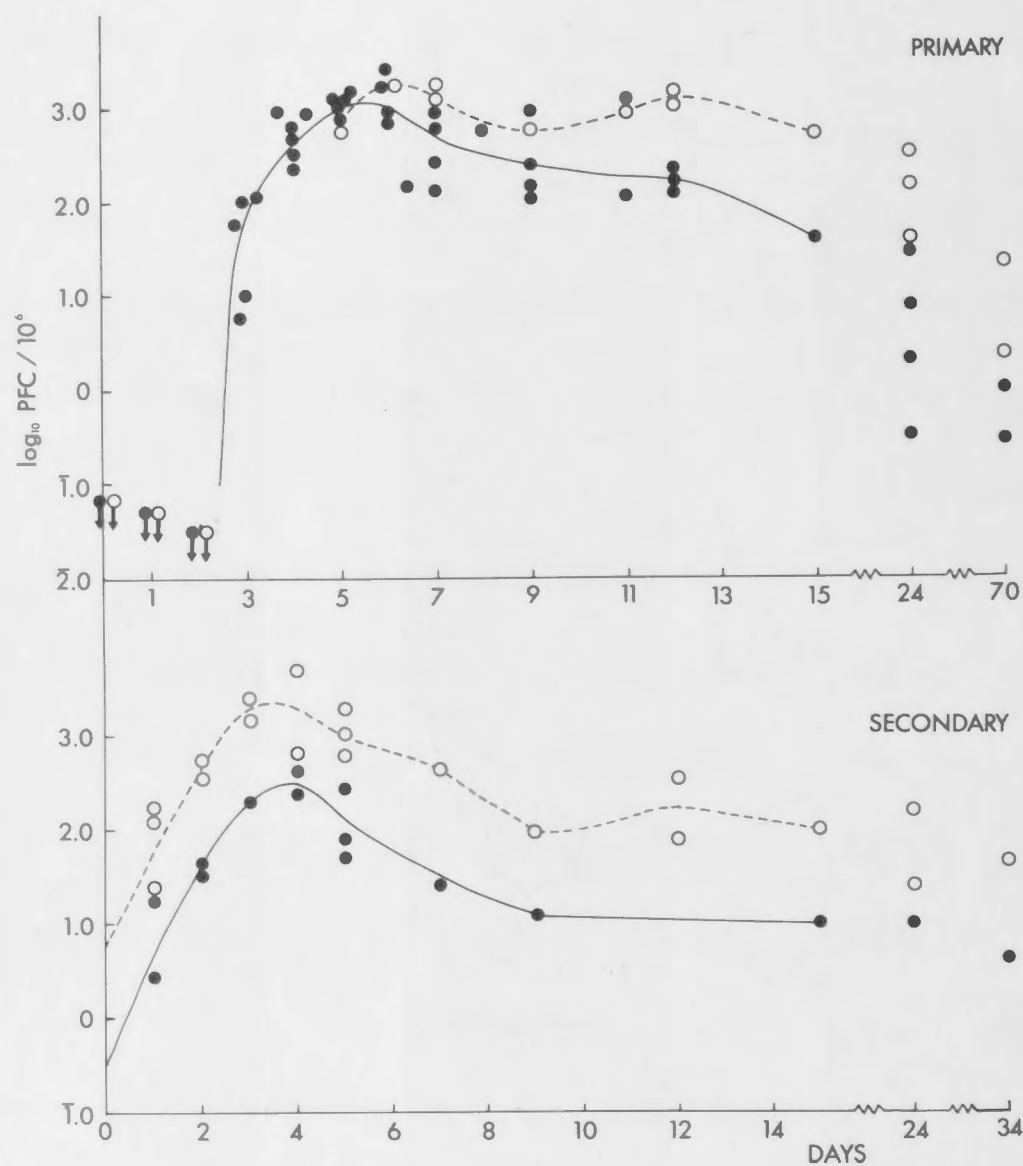


Fig. 5.2.

Numbers of plaque-forming cells in the popliteal lymph nodes of adult CBA mice at different times after immunization with sheep erythrocytes. Each point represents 4 pooled nodes from 2 mice. Black circles; cells producing 19S antibody. Open circles connected by broken line; 7S antibody producers.



D. NUMBERS OF ANTIBODY-FORMING CELLS IN THE  
POPLITEAL NODES AT DIFFERENT TIMES AFTER ANTIGENIC  
STIMULATION.

The changing response with time of mouse popliteal nodes is shown in fig. 5.2. Each point represents a pool of 4 nodes from 2 adult CBA mice (10-18 weeks old, male or female). Immunizing doses ranged from  $1-5 \times 10^8$  sheep erythrocytes per footpad.

In the primary response a striking lag phase of nearly 3 days is seen. No plaque-forming cells can be found during this time, even when the whole node is plated out, stroma and cells together. An extremely rapid rise in numbers of cells forming 19S antibody follows, reaching a peak at 4-5 days, when <sup>cells</sup> producing 7S antibody first appear. Numbers of 7S-producers are at a maximum around 11 days after antigenic stimulation, when they are about 10 times as numerous as cells releasing antibody of high haemolytic efficiency. This 10-fold disparity between cells of the 2 types is preserved throughout the remainder of the primary response, and for the entire course of the secondary response. Plaque-forming cells of both kinds persist in the stimulated node; significant numbers may still be found 70 days after antigenic stimulation.

The secondary response begins without obvious delay, and peak numbers of active cells appear more quickly than in the primary response. Immunological memory for both 19S and 7S antibody production exists in this system. Cells producing antibody of low haemolytic efficiency always predominate in a secondary response; some overlap of points may be seen in fig. 5.2, but in any 1 mouse, the ratio 7S/19S plaques was about 10/1.

#### E. MORPHOLOGY OF THE ANTIBODY-FORMING CELLS.

##### I. Methods.

Mice received an injection of  $2 \times 10^8$  sheep red cells in both footpads. Secondary injections were given 1-3 months after primaries. At intervals after stimulation, lymph node cell suspensions were assayed for antibody-forming cells in microdrops under oil. Plaque-forming cells were manipulated out of the microdrops, smeared individually and stained with Leishman's as described in chapter 3. The 2-step method was used when proportions of active cells were small.

To identify cells releasing 7S antibody, anti-globulin was added to the medium. At all times when

TABLE 5.7

Classification of antibody-forming cells.

Name		diameter	basophilia	<u>nucleus</u> cytoplasm ratio
blasts		≥16	1-4	≥0.5
basophilic mononuclears	large	13-15	≥2	>0.5
	medium	11-12	≥2	>0.5
	small	<11	≥2	>0.5
lymphocytes	large	13-15	<2	>0.5
	medium	11-12	<2	>0.5
	small	<11	<2	>0.5
plasma cells	large	≥12	≥2	≤0.5
	small	<12	≥2	≤0.5

cells producing 7S antibody were selected, the proportion of 19S-producers in the lymph node cell suspensions was less than 10%; the population of "7S-producers" described below could have been contaminated with up to 10% of cells releasing 19S antibody.

## II. Classifying cells.

Three main morphological features were considered in classifying these cells; size, basophilia, and ratio of nucleus to area of the whole cell (N/C ratio). The average diameter of cells was measured with an eyepiece graticule. The degree of basophilia was expressed on a scale from "1" (pale staining) to "4" (very dark). N/C ratio was estimated by eye. In control experiments, pieces of paper were cut to simulate nuclei and cell outlines, then estimates of relative areas were checked by weighing the paper. It was found that the N/C ratio could be estimated to within 10% in most cases.

Table 5.7 shows the main cell types distinguished. Lymphocytes, defined by their pale staining, were arbitrarily divided into the 3 usual groups, large, medium and small. Three similar classes of "basophilic mononuclear" cells were distinguished from lymphocytes by their greater basophilia. Plasma cells were basophilic, with a nucleus occupying not more than half of the total area of the cell, and usually eccentrically placed.



TABLE 5.8

Range of diameters and nucleus/cytoplasm ratios in 17 cells producing 19S antibody, and 17 producing 7S antibody, taken from the same mouse 5 days after secondary antigenic stimulation.

Cell no.	19S population		7S population	
	Diameter	N/C	Diameter	N/C
1	12	0.7	13	0.4
2	12	0.6	13	0.5
3	11	0.65	13	0.4
4	12	0.4	11	0.6
5	11	0.7	12	0.3
6	11	0.6	12	0.7
7	12	0.7	14	0.6
8	12	0.7	12	0.4
9	9	0.8	12	0.6
10	10	0.5	10	0.7
11	12	0.45	11	0.7
12	13	0.6	14	0.6
13	13	0.6	13	0.4
14	13	0.6	12	0.7
15	11	0.7	11	0.7
16	14	0.8	14	0.55
17	11	0.6	10	0.55

Blasts were simply defined as very large antibody-forming cells; they were nearly all moderately basophilic.

### III. Results.

285 plaque-forming cells have been examined from mouse lymph nodes. In addition, 10 plaque-forming cells have been obtained from the spleens of mice which had not been intentionally immunized. Figures 5.3 and 5.4 show the morphological classifications of all the active lymph node cells. Fig. 5.5 illustrates the range of antibody-forming cell types produced by immunization, while fig. 5.6 shows plaque-forming cells from "normal" mice.

#### (a). 19S/7S comparison.

No striking differences between the populations of cells releasing 19S or 7S antibody have emerged (figs. 5.3 and 5.4). Certainly there is no monopoly of any particular morphological type by either functional class. The most detailed comparison available for the 19S and 7S-producing cells from 1 mouse at the same time is presented in table 5.8. There was no significant difference in N/C ratio ( $p = 0.1$ ) or average size ( $p = 0.3$ ) between these 2 groups, and the degree of basophilia was the same (about 2.5 on the subjective

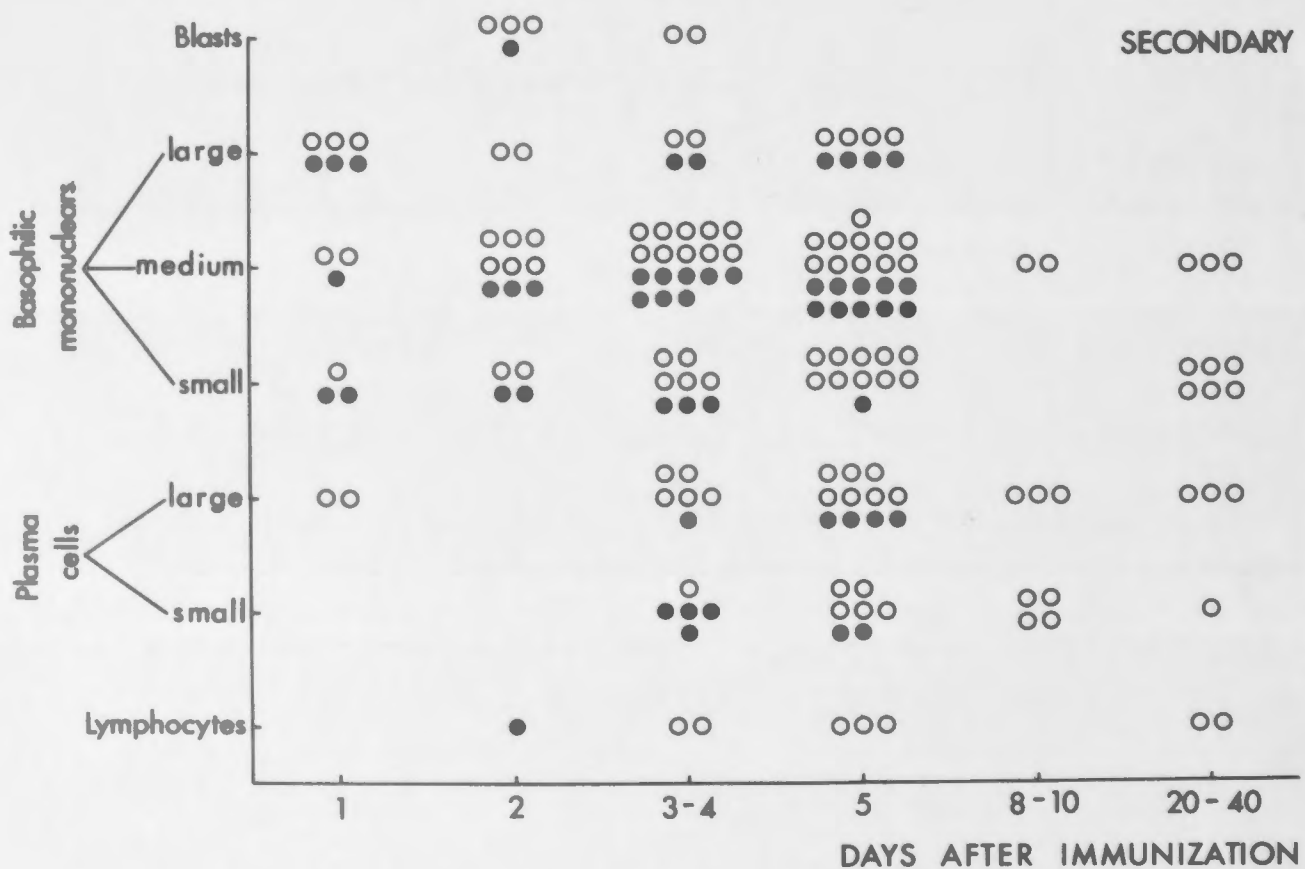
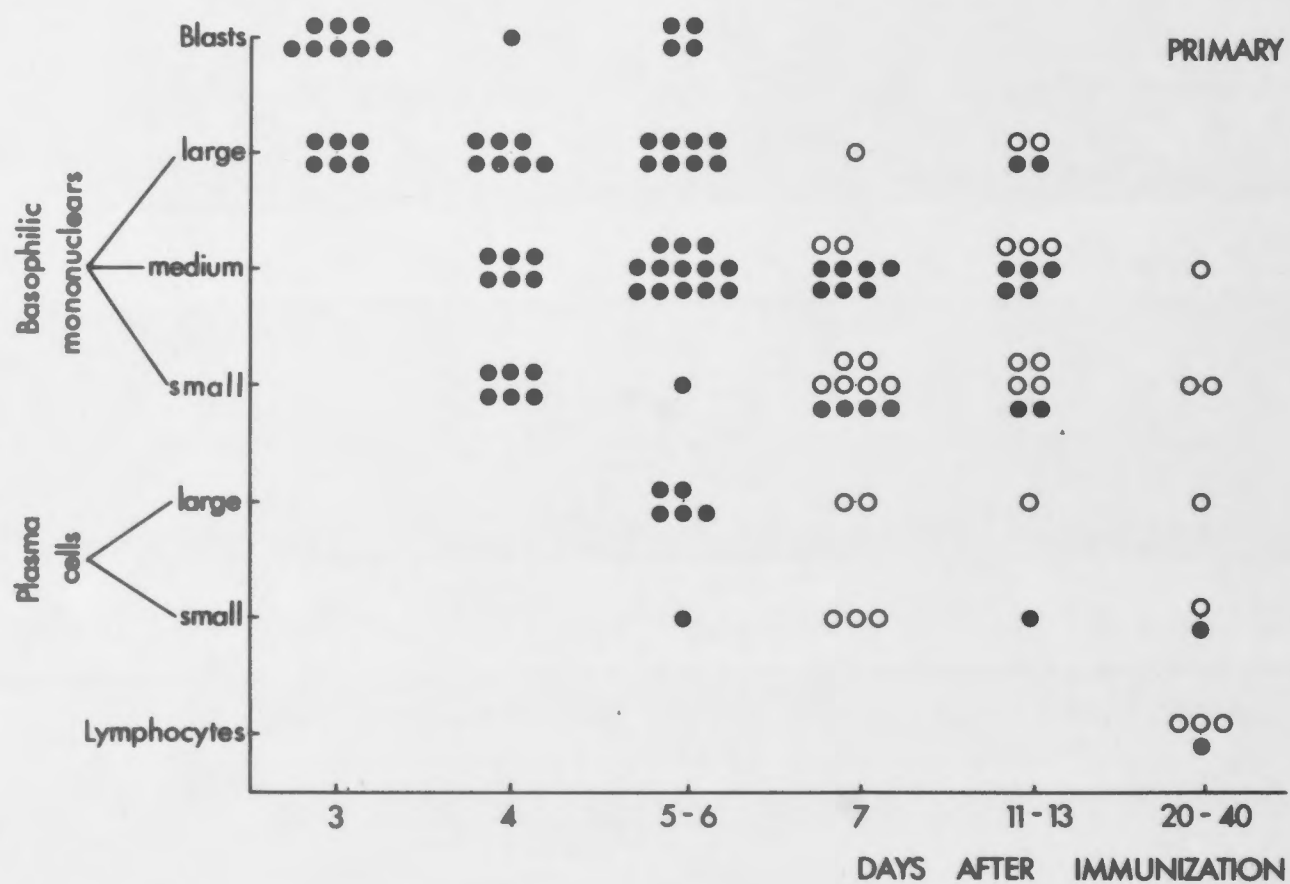


Fig. 5.3. and 5.4.

Plaque-forming cells from popliteal lymph nodes of mice. Each circle represents 1 cell. Black circles; cells releasing 19S antibody. Open circles; cells releasing 7S antibody.

scale) in almost all the cells.

(b). Predominant cell types.

Most of the antibody-forming cells were "basophilic mononuclears" of various sizes (fig. 5.5i to 5.5r). Their average size decreased as the response progressed. Plasma cells were less common (a total of 56 in 285 cells); more plasma cells were found in secondary than in primary responses.

(c). Early stages of response.

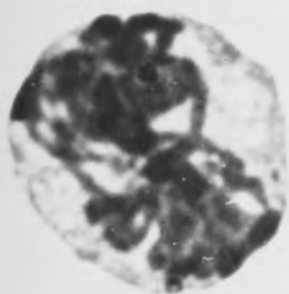
In the primary response, the earliest antibody-forming cells to appear were all blasts or large basophilic mononuclears. In the secondary, blasts were rare, and the earliest antibody-formers were large, medium or small basophilic cells.

(d). Middle phase of the response.

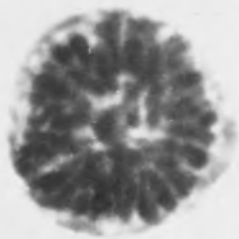
During the period in which active cells were most plentiful (4-7 days after antigen in the primary, 3-5 in the secondary response), the main antibody-forming cell type was a basophilic mononuclear from 9-13 $\mu$  in diameter, with a nucleus occupying from 0.6 to 0.8 of the total area of the cell. Plasma cells, as defined here, were most common at the peak of the secondary response, when they represented about 1/4 to 1/3 of the active cells.



Fig. 5.5. Plaque-forming cells from the popliteal lymph nodes of immunized mice. (a) and (b): cells in mitosis; (c) and (d): blasts; (e) and (f), (g) and (h): large and small plasma cells; (i) - (r): basophilic mononuclears of different sizes; (s) and (t): small lymphocytes. The type of antibody-forming cell most commonly found was the "medium basophilic mononuclear" (k) and (l). The black and white photographs can give only an approximate indication of the degree of basophilia of a cell. For example, (m) was considerably more basophilic than (s). Magnification approx. 1800.



a



b



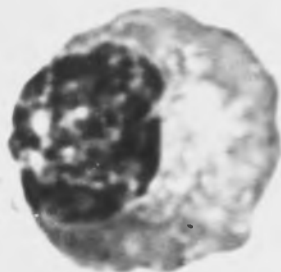
c



d



e



f



g



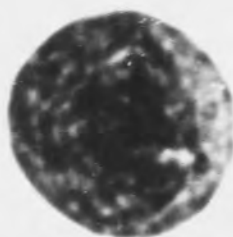
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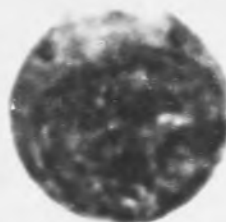
i



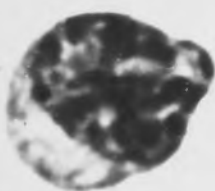
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k



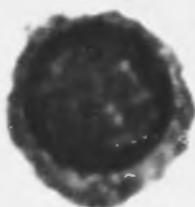
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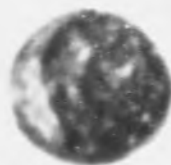
m



n



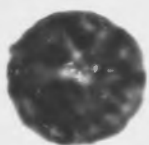
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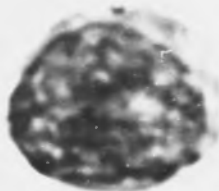
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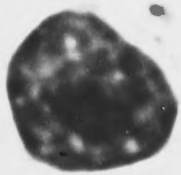


s



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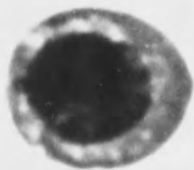
Fig. 5.6. Plaque-forming cells from the spleens of non-immunized mice. (a): small - medium lymphocyte; (b): a similar cell, but more basophilic than (a), classified as a medium basophilic mononuclear; (c) and (d): atypical plasma cells ?; (e): 2 small lymphocytes which together produced 1 plaque; (f): medium - large lymphocyte. Magnification approx. 1800.



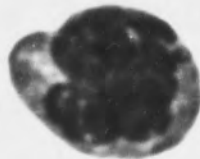
a



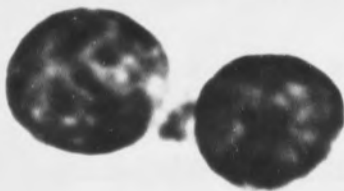
b



c



d



e



f



(e). Late stages of response.

Active cells were difficult to isolate at these times. Of those examined, most were very small, and a number were small to medium lymphocytes. Some had little cytoplasm; the cell in fig. 5.5r was specially noticed, in the living state, as being not much larger than a sheep red cell, yet it formed a large plaque.

(f). Plaque-forming cells from unimmunized mice.

Seven plaque-forming cells were isolated by the 2-step procedure. In addition, 3 pairs of joined cells, each responsible for 1 plaque, were successfully smeared, when it was found that the 2 members of each pair were morphologically almost identical; each pair was therefore scored as 1 active cell, since it could not be decided whether 1 or both members of the pair were releasing antibody. Several other pairs were found to involve 2 morphologically dissimilar members - these have not been included in the results.

Of the 10 plaque-forming cells, 6 were non-basophilic lymphocytes, 1 large, 1 medium and 4 small. Two of the remaining 4 cells were classed small basophilic mononuclears, and 2 as small plasma cells, although these had more or less central nuclei and were not at all typical in appearance (fig. 5.6).

The antibody-forming cells in normal mice seem to resemble the population of active cells taken from immunized lymph nodes at a late stage in an immune response. This supports the idea that plaque-forming cells in unimmunized animals arise by previous stimulation with cross-reacting antigens. Such antigens would have to be of a type which elicits only 19S antibody production (bacterial endotoxins?), since cells releasing 7S haemolysin do not seem to occur in normal spleens. The plaques produced by these cells were just as big as those formed by cells at any time during an intentionally induced immune response.

globulin serum used to develop plaques enhanced the lytic effect of 7S but not 19S antibody. Third, 19S antibody is believed to be several hundred times more efficient at lysing red cells than 7S antibody (Humphrey and Pouroushain, 1965; Horace and Rapp, 1965; Heller and Wigzell, 1965).

It should be explained that the classification of cells as producers of 19S or 7S antibody rests only on a correlation between a property of the haemolysin they release and the same property of antibodies found in serum. Serum contains the products of a very large number of cells; small subclasses of haemolysin may exist which have very different properties from the

## F. DISCUSSION.

Antigenic stimulation of the popliteal lymph nodes of mice with sheep erythrocytes gives rise to 2 classes of antibody-forming cells; these are distinguished by their ability to form plaques in the absence or presence of anti-mouse gamma globulin antiserum. Three observations suggest that these cells are releasing 19S and 7S haemolysin respectively. First, the sequential appearance of antibody-forming cells of the 2 classes correlates well with the serum titres of 19S and 7S haemolysin, as described by others. Second, the anti-globulin serum used to develop plaques enhanced the lytic effect of 7S but not 19S antibody. Third, 19S antibody is believed to be several hundred times more efficient at lysing red cells than 7S antibody (Humphrey and Dourmashkin, 1965; Borsos and Rapp, 1965; Moller and Wigzell, 1965).

It should be emphasised that the classification of cells as producers of 19S or 7S antibody rests only on a correlation between a property of the haemolysin they release and the same property of antibodies found in serum. Serum contains the products of a very large number of cells; small subclasses of haemolysin may exist which have very different properties from the

average. For example, a class of 7S antibody may exist which lyses red cells with the efficiency of most 19S molecules. Cells producing this sort of antibody would be wrongly classified. Formal proof of the molecular weight of the antibody from a single cell would require ultracentrifugation of the product of that cell alone.

The kinetics of appearance of cells producing 19S antibody in the spleens of mice after immunization with sheep erythrocytes has been well studied since the first description by Jerne et al. (1963). The response of the popliteal lymph node differs in several ways. No plaque-forming cells can be found in the node for nearly 3 days after antigenic stimulation. A period follows in which active cells rapidly appear, doubling their numbers every 2-3 hours, on an average. This rate of increase is incompatible with any known capacity of lymphoid cells for mitotic division; presumably cells are being "switched on" during this phase. By contrast, the lag phase is much shorter in the spleen, around 24 hours, (Wigzell et al. 1966), and plaque-forming cells appear more slowly, to reach peak numbers at about 4 days after antigenic stimulation. Plaque-forming cells can usually be found in the spleens of "normal" mice, while lymph



nodes do not seem to harbour such cells.

Cells producing 19S antibody fall off as those releasing 7S haemolysin increase in number. Moller and Wigzell (1965) have suggested that the 7S antibody "switches off" recruitment of 19S precursors. The situation seems to be more complex than this however, since cells of both types persist for at least 70 days after primary stimulation, and eventually show a more or less parallel decline in numbers. During the secondary response, 19S and 7S plaque-forming cells both rise and fall in parallel (fig. 5.2). No doubt homeostatic mechanisms control the proliferation of antibody-forming cells; antibody, and persisting antigen may both be involved.

The majority of the haemolysin-producing cells in mice were found to be basophilic, from 8-14 $\mu$  in diameter and with a nucleus which occupied most of the apparent area of the smeared cell. These were called, non-committally, "basophilic mononuclears". Some of the larger members of this group may correspond to the immature plasma cells described by others. The smaller cells would probably be indistinguishable from normal small to medium lymphocytes in preparations where basophilia was not apparent, for example in phase contrast pictures of living cells, or in fluorescence-

stained sections or smears. A number of apparently "normal" non-basophilic small lymphocytes were found to be producing antibody, especially late in immune responses, or in samples from the spleens of animals which had not been deliberately immunized.

Evidently, antibody formation is not the prerogative of mature plasma cells in this system. The immediate question which the morphological heterogeneity of antibody-forming cells poses is; how are these different types interrelated? To answer this, investigations using markers additional to morphology and function are required.

by light microscopy at all stages of the immune response. A wide variety of morphological types was found to produce hemolytic antibody. The predominant type of active cell was basophilic, and from 5-14  $\mu$  in diameter, with a relatively large nucleus. Mature plasma cells were found, but less commonly. No consistent differences were observed between cells releasing 15S and 7S antibody.

Ten plaque-forming cells from the spleen of unimmunized mice have been characterized. Six of these were lymphocytes, 2 were small basophilic cells, and 2 were classified as atypical plasma cells.

G. SUMMARY.

Methods have been standardized for detecting 2 classes of haemolysin-producing cell in the mouse, and evidence is presented that these cells are releasing either 19S or 7S antibody. A description is given of the changing numbers of cells of both types in popliteal nodes of mice throughout the primary and secondary immune response to sheep erythrocytes.

Individual antibody-forming cells have been examined by light microscopy at all stages of the immune response. A wide variety of morphological types was found to produce haemolytic antibody. The predominant type of active cell was basophilic, and from 8-14 $\mu$  in diameter, with a relatively large nucleus. Mature plasma cells were found, but less commonly. No consistent differences were observed between cells releasing 19S and 7S antibody.

Ten plaque-forming cells from the spleens of unimmunized mice have been characterized. Six of these were lymphocytes, 2 were small basophilic cells, and 2 were classed as atypical plasma cells.

## A. INTRODUCTION.

It is well known that the injection of a single antigen into an animal results in the appearance of serum antibody which is heterogeneous in such properties as molecular weight, electrophoretic mobility, antigenicity (chemical structure) and specificity. At the cellular level, an obvious question is: does each cell produce all these types of antibody, or only 1 or a restricted number of types? Results of fluorescence studies (CHAPTER 6. chapter 1) indicate that antibodies of different broad chemical groups are usually found in the same cell. This is in contrast to the previous chapter NATURE OF THE ANTIBODY PRODUCED BY SINGLE CELLS. subdivision of cells on the basis of the haemolytic efficiency (and hence probably molecular weight) of the antibody produced. But the most characteristic property of antibody is its specificity, and the range of specificity to be found in the antibody from a single cell is of most relevance to immunological theory. Cells from animals immunized with more than 1 antigen usually produce antibody of only 1 detectable specificity (Kessal, 1958), but reports of 2 antibody specificities in single cells have been published over the last few years (see chapter 1).

This chapter demonstrates heterogeneity of antibody



## A. INTRODUCTION.

It is well known that the injection of a single antigen into an animal results in the appearance of serum antibody which is heterogeneous in such properties as molecular weight, electrophoretic mobility, antigenicity (chemical structure) and specificity. At the cellular level, an obvious question is; does each cell produce all these types of antibody, or only 1 or a restricted number of types? Results of fluorescence studies (discussed in chapter 1) indicate that antibodies of different broad chemical groups are usually found in different cells. The work cited in the previous chapter demonstrates a subdivision of cells on the basis of the haemolytic efficiency (and hence probably molecular weight) of the antibody produced. But the most characteristic property of antibody is its specificity, and the range of specificity to be found in the antibody from a single cell is of most relevance to immunological theory. Cells from animals immunized with more than 1 antigen usually produce antibody of only 1 detectable specificity (Nossal, 1958), but reports of 2 antibody specificities in single cells have been published over the last few years (see chapter 1).

This chapter demonstrates heterogeneity of antibody

TABLE 6.1

Plaque-forming cells in the popliteal efferent lymph of 2 sheep, immunized with Salmonella muenchen and tested against sheep erythrocytes sensitised with various lipopolysaccharides. (Lymph collected from each sheep 4 days after primary injection of S. muenchen).

Sheep A.

Source of bacterial antigen used to sensitize rbc's.	Group of bacterium	O-antigens	plaque-forming cells/10 <sup>6</sup>	95% conf. limits. ±
<u>S. muenchen</u>	C <sub>2</sub>	6,8	4525	1800
<u>S. bovis-morbificans</u>	C <sub>2</sub>	6,8	2740	1530
<u>S. anatum.</u>	E <sub>1</sub>	3,10	2225	1005
<u>S. abortus-bovis</u>	B	1,4,12,27	530	400
<u>S. typhimurium</u>	B	1,4,5,12	100	only 1 plaque
<u>E. coli</u>	-	-	< 50	
Normal sheep red cells	-	-	< 50	

Sheep B.

<u>S. muenchen</u>	C <sub>2</sub>	6,8	19300	4100
<u>S. bovis-morbificans</u>	C <sub>2</sub>	6,8	17400	4000
<u>S. anatum</u>	E <sub>1</sub>	3,10	50	only 1 plaque.
<u>S. abortus-bovis</u>	B	1,4,12,27	4900	1260

produced by different cells when 1 antigenic particle is injected, and homogeneity of the antibody from single cells when 2 antigens are injected simultaneously.

B. HETEROGENEITY OF ANTIBODY SPECIFICITY: BETWEEN CELLS.

All the sheep used for the work described in chapter 4 were antigenically stimulated by subcutaneous injection of boiled Salmonella muenchen. Popliteal efferent lymph was then assayed for cells producing plaques when incubated among erythrocytes sensitised with a lipopolysaccharide extract of the homologous bacteria. In 2 sheep, further samples of the same efferent lymph cells were tested for their ability to lyse sheep red cells coated with lipopolysaccharides from other bacteria.

Table 6.1 shows that smaller numbers of cells were able to lyse erythrocytes coated with lipopolysaccharides from other salmonellae. Antigens other than the O-antigens of the Kauffman-White scheme must have been responsible for this effect. Cells from the 2 sheep showed different patterns of cross-reactivity. Erythrocytes sensitised with lipopolysaccharide from Salmonella anatum detected almost as many plaques as did erythrocytes coated with homologous antigen in the

case of sheep A. By contrast, very few cells from sheep B were able to lyse erythrocytes coated with S. anatum lipopolysaccharide.

A maximum number of plaque-forming cells could be detected when the homologous lipopolysaccharide was used to sensitise target erythrocytes. Presumably the cells reacting against heterologous lipopolysaccharides represent a fraction of this total population of antibody-forming cells. The alternative, that the use of homologous and heterologous antigens detects entirely separate populations, was disproved in the following experiment.

Cells from sheep B, collected 1 day later than those tested for table 6.1, were found to contain 1450 cells per million active against S. muenchen lipopolysaccharide, and 270 per million active against S. anatum. (The ratio of the numbers of these 2 populations was thus the same as on the previous day). Lymph cells were then incubated in chambers with a mixture of red cells, half of which had been sensitised with the homologous, and half with the heterologous, lipopolysaccharide. Of 33 plaques, 11 were "clear", indicating lysis of both sets of red cells, and 22 were "partial" where only 1 of the lipopolysaccharides had reacted with antibody. Partial plaques were less easily detected than clear



ones, and a few may have been missed. The results fit the interpretation that those cells producing antibody which combined with the heterologous antigens also formed antibody to the homologous lipopolysaccharides. Whether this double reactivity was due to 2 distinct antibodies of different specificities, or to one which cross reacted with both antigens could not be decided from the data available (see "Discussion").

#### C. HETEROGENEITY OF ANTIBODY SPECIFICITY: WITHIN CELLS.

In an animal immunized with 2 antigens, the incidence of cells producing 2 antibodies might be greatly increased if both antigens were presented on the same particle. To test this idea, adult CBA mice were stimulated with 2 non-cross reacting antigens coupled to the same or to different individual red cells.

##### (a). Lack of cross reaction between antigens.

The 2 antigens used were lipopolysaccharides from Salmonella muenchen and Shigella sonnei. Cells from mice immunized with either of these antigens did not produce plaques when incubated with sheep erythrocytes sensitised with the other lipopolysaccharide.

##### (b). Sensitising isologous red cells.

Mouse erythrocytes were sensitised by incubating

TABLE 6.2

Lytic titres of 2 rabbit antisera prepared against Salmonella muenchen (A), or Shigella sonnei (B), and tested against sheep or mouse red cells sensitised with A, B, or A and B lipopolysaccharides.

Rabbit antiserum	Type of red cell sensitised	Sensitising lipopolysacc.	50% haemolytic end-point (recip. of dilution).
A	sheep	A	35,600
A	sheep	B	< 200
A	sheep	A+B	60,800
B	sheep	A	< 10
B	sheep	B	130,000
B	sheep	A+B	60,800
A	mouse	A+B	160
B	mouse	A+B	80

0.1ml of the packed cells with 2 ml saline containing 400µgm/ml of the lipopolysaccharide (see chapter 2). For double sensitization, both antigens were incubated together with the erythrocytes. It was shown that each individual red cell had both antigens on its surface by the following 2-step procedure:

(i). Two antisera, prepared in rabbits against the lipopolysaccharides, were shown to be specific for the immunizing antigen by testing against sheep red cells coated with either lipopolysaccharide (table 6.2).

(ii). Each of these specific antisera lysed all the mouse red cells in a doubly sensitised population. Titres were rather low - the mouse cells seem to be relatively resistant to lysis. Sheep red cells doubly sensitised in the same way lysed to high titre with either antiserum. (table 6.2).

(c). Immunizing mice.

Six mice were immunized in 3 groups of 2. Each received 0.025ml of sensitised isologous erythrocytes intravenously (lateral tail vein). All mice were killed 4 days later, and single cell suspensions made from the 2 pooled spleens of each group.

Group D (doubly sensitised). These 2 mice received red cells sensitised so that each cell had both antigens on its surface.

TABLE 6.3

Plaque-forming cells from the spleens of mice immunized with 2 antigens; tested against sheep erythrocytes coated with only 1 of the antigens (see text). As in table 6.2, "A" refers to antigen from Salmonella muenchen, and "B" to antigen from Shigella sonnei.

Spleen pool.	Sheep eryths. sensitised with:	Plaque-forming cells/ $10^6$ .
D	A	100
D	B	70
S	A	300
S	B	100
C	A	120
C	B	150



Group S (separately sensitised). Half of red cells had been sensitised with 1 antigen, and half with the other, then the 2 batches were mixed before injection.

Group C (control). One mouse received 0.025ml cells sensitised with lipopolysaccharide from S. muenchen, and the other, the same number sensitised with an extract from Shigella sonnei. Spleen cells were pooled after killing the mice.

(d). Assaying for plaque-forming cells.

The spleen cells from each pool were first tested against populations of sheep red cells sensitised with only 1 antigen (table 6.3). Each pool was then assayed, in standard chambers, against a mixture of sheep erythrocytes, half of which had been sensitised with 1 antigen, and half with the other. About 300 plaques were examined for each pool, to record numbers of "probable" and "possible" clear plaques formed by lysis of both sets of erythrocytes. The concentration of spleen cells was adjusted to give about 40 plaques per chamber so as effectively to eliminate overlapping of plaques. All readings were done "blind".

(e). Results.

Clear plaques were uncommon, and their frequency was statistically indistinguishable between groups

TABLE 6.4

Double plaques when cells from the 3 spleen pools were assayed against a mixture of sheep erythrocytes, half of which had been sensitised with antigen A, and half with B. (See text).

Spleen pool	<u>Double plaques counted.</u>		
	Possible	Probable	Total
D	2	2	4
S	6	0	6
C	4	4	8

(table 6.4). It seems likely that the areas of double lysis observed were due to a background of cells producing antibody to sheep erythrocytes. The control group would also have detected double plaques caused by cross-reacting antibody, overlapping, or mechanical disturbance of the monolayers.

(f). Conclusions from this experiment.

No spleen cells releasing haemolytic antibodies of 2 different specificities were detected when mice were doubly immunized. The result was the same when the 2 antigens were attached either to the same, or to different individual erythrocytes.

Recently, Hiramoto and Smith (1961) reported that in guinea-pigs immunized with gamma-globulin, only half the antibody-forming cells could be shown, by double fluorescence, to be producing separate antibodies against 2 fractions of the antigen molecule. The difference in result between this and other reports is probably one of resolution; if Hiramoto and Smith had used whole gamma globulin instead of fractions to test

#### D. DISCUSSION.

This brief study demonstrates the usefulness of the plaque technique for analysing the antibody produced by single cells. Two main points have been shown. First, when a complex antigen was injected into a sheep, active cells differed in the specificity of antibody which they released. Second, when mice were injected with 2 antigens simultaneously, the antigen being attached either to the same or to different individual isologous erythrocytes, the great majority of cells released antibody possessing only 1 of the 2 detectable specificities. Neither of these findings is new. That individual cells from doubly stimulated animals usually produce antibody directed against only 1 of the antigens was first shown by Nossal (1958), and the corollary, that different cells produce different antibodies was directly demonstrated by Makela (1964).

Recently, Hiramoto and Hamlin (1965) reported that in guinea-pigs immunized with gamma-globulin, nearly half the antibody-forming cells could be shown, by double fluorescence, to be producing separate antibodies against 2 fractions of the antigen molecule. The difference in result between this and other reports is probably one of resolution; if Hiramoto and Hamlin had used whole gamma globulin instead of fractions to test



their cells, they would have concluded that all the cells were releasing only 1 antibody, directed against gamma globulin. In the present study, cells detected as forming antibody to one lipopolysaccharide and not to another may well have been releasing a number of antibodies of different specificities corresponding to different portions of the lipopolysaccharide molecule.

Evidently, a much sharper resolution of the specificity of the antibodies involved is needed before any upper limit can be set to the number of different specificities which a single cell may produce. Stated in another way, the problem is to find how large a portion of the antigen influences the cell, and how antibody combining sites against this antigenic fragment are distributed among the separate molecules coming from the cell. If an antigen molecule with recognizable determinants A, B and O is injected, what proportion of cells reacting against A also react with B, and with O, and is this reactivity on separate molecules?

Such an analysis may be technically feasible, and the plaque test, with its capacity for rapid scanning of a large number of active cells, would seem to be the most suitable of available methods. "A", "B" and "O" could be enterobacterial antigens or, much better, haptens, using Merchant and Hraba's (1966) adaptation

of the plaque technique. The first step would be to immunize an animal with 2 antigenic determinants, A and B, joined together as 1 molecule, "AB". For immunization, AB could be attached to a protein carrier or to isologous erythrocytes. The lymphoid cells of the immunized animal would then be tested against a mixed population of erythrocytes conjugated separately with A or B. If Hiramoto and Hamlin are right, most active cells would produce clear plaques. Plaques due to the release of 1 antibody (anti-AB) would presumably be abolished by the presence of either free antigen A or B in the medium. (Preliminary tests have shown that plaque lysis of lipopolysaccharide-coated erythrocytes can be inhibited by free lipopolysaccharide in the medium). Plaques due to separate anti-A plus anti-B should be converted from clear to partial by either 1 inhibitor, and abolished by both together. This process has not yet been tested with plaque-forming cells. It would depend on the same principle as the procedure of cross-absorption which is commonly used to distinguish cross-reacting from separate antibodies in serum.

The next step would be to immunize an animal with AOB, where O is of variable size, its function being to separate A and B. The test for antibody-forming

cells would then be performed in exactly the same way, when antibody capable of combining with the whole determinant AOB could be recognized. If A, O and B were available separately and in pairs for combination with red cells, the population could be further analysed for cells producing antibodies to A, O, B, AO, AB, and OB, and for combinations of these. In this way it might be possible to determine the amount of antigen "seen" by the antibody-forming cell, and the range of antibody specificities produced.

*Salmonella typhimurium* was injected into sheep, and antibody of different specificity was released by different individual cells from popliteal lymph. When *Salmonella typhimurium* and *Shigella lipopolysaccharides* were injected simultaneously into mice, cells producing antibodies against both these antigens were not detected, and must have comprised less than 2% of the population of antibody-forming cells. This result was obtained even when both antigens were coupled to the same individual red cells before immunization.

A method is suggested for attempting to determine the size of antigen fragments which influence the specificity of antibody released by single cells, and for analysing the distribution of combining sites among the separate molecules coming from a cell.

E. SUMMARY.

A mixed plaque technique has been used for analysing the antibody specificities produced by single cells. Batches of target erythrocytes are coated with only 1 of the 2 immunizing antigens; equal numbers of each sort of target cell are then incubated in monolayers with the lymphoid cells under test. Cells releasing antibodies of one or both specificities produce partial and clear plaques respectively. When a complex antigen, boiled Salmonella muenchen, was injected into sheep, antibody of different specificity was released by different individual cells from popliteal efferent lymph. When Salmonella and Shigella lipopolysaccharides were injected simultaneously into mice, cells producing antibodies against both these antigens were not detected, and must have comprised less than 2% of the population of antibody-forming cells. This result was obtained even when both antigens were coupled to the same individual isologous red cells before immunization.

A method is suggested for attempting to determine the size of antigen fragments which influence the specificity of antibody released by single cells, and for analysing the distribution of combining sites among the separate molecules coming from a cell.



Methods which have been used to trace the life history of cells involved in antibody formation were reviewed in chapter 1. Two of the most promising model systems appeared to be the *in vitro* culturing of cells, and the *in vivo* colony method of Kennedy et al. (1966), and Playfair et al. (1969). This chapter records some relatively unsuccessful attempts to culture antibody-forming cells *in vitro*, and a more thorough exploration of the *in vivo* colony method.

## CHAPTER 7

### 1. *IN VITRO* CULTURES.

#### MODEL SYSTEMS FOR STUDYING THE DEVELOPMENTAL HISTORY OF ANTIBODY-FORMING CELLS.

cells were incubated for up to 1 day. Also unsuccessful were attempts to culture single antibody-forming cells in microdrops of medium which had been "conditioned" by preincubation with normal spleen cells. Cells stopped releasing detectable antibody within 24 hours, and were never seen to divide in the microdrops. The ability to produce plaque was retained for longer periods by a small proportion of spleen cells in mass cultures. Fig. 7.1 shows the results of 1 experiment. Suspensions of dispersed spleen cells

Methods which have been used to trace the life histories of cells involved in antibody formation were reviewed in chapter 1. Two of the most promising model systems appeared to be the in vitro culturing of cells, and the in vivo colony method of Kennedy et al. (1966), and Playfair et al. (1965). This chapter records some relatively unsuccessful attempts to culture antibody-forming cells in vitro, and a more thorough exploration of the in vivo colony model.

#### A. IN VITRO CULTURES.

Plaques did not develop when standard chambers containing normal mouse spleen cells and sheep red cells were incubated for up to 3 days. Also unsuccessful were attempts to culture single antibody-forming cells in microdrops of medium which had been "conditioned" by preincubation with normal spleen cells. Cells stopped releasing detectable antibody within 24 hours, and were never seen to divide in the microdrops.

The ability to produce plaques was retained for longer periods by a small proportion of spleen cells in mass cultures. Fig. 7.1 shows the results of 1 experiment. Suspensions of dispersed spleen cells

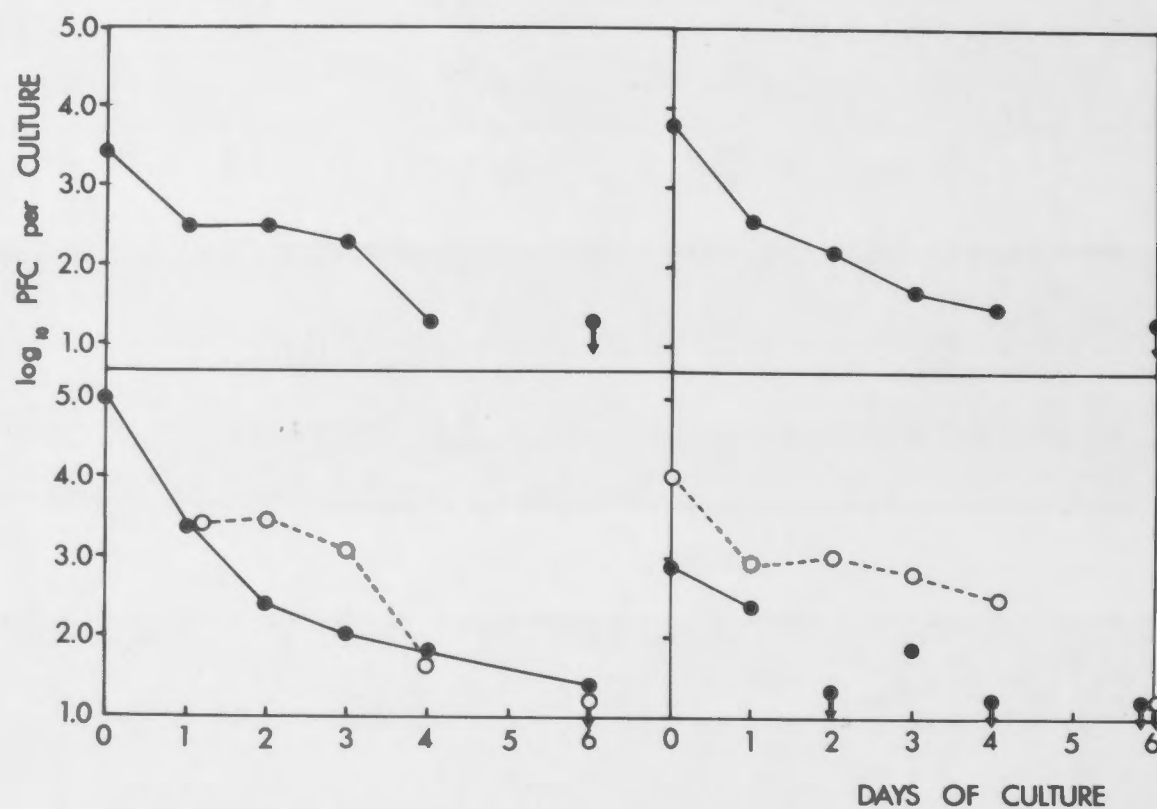


Fig. 7.1

Survival of plaque-forming cells in cultures in vitro. The ordinate is the logarithm of numbers of plaque-forming cells found in replicate culture dishes at different times. Unbroken lines represent cells producing 19S antibody, broken lines follow numbers of cells forming 7S antibody.

Top left: Primary immunization 2 days before removing spleen cells for culture.  
 Top right: Primary, 3 days before.  
 Bottom left: Primary 4 days before.  
 Bottom right: Secondary, 2 days before.

were prepared from 4 groups of mice which had received a primary intravenous injection of sheep erythrocytes 2, 3 or 4 days previously, or a secondary injection 2 days before sacrifice. Cells from each group were incubated in 6 small (5cm diameter) glass petri dishes in an atmosphere of 5% CO<sub>2</sub>. Each petri dish contained  $2.5 \times 10^7$  nucleated cells in 7.5ml of Eagle's medium with 10% added foetal calf serum, and 0.04% sodium bicarbonate. This medium was changed daily. The cells from 1 dish in each group were counted every day, tested for viability by trypan blue exclusion, and assayed for plaque-forming cells by the free suspension technique.

During the first 24 hours of incubation, the number of antibody-forming cells dropped rapidly in all groups (fig. 7.1). For the next 48 hours, numbers of cells releasing antibody of high haemolytic efficiency (probably 19S antibody), declined more slowly, except in cultures from mice which had been immunized for the first time 2 days before killing, where the low level of active cells was maintained. Plaques due to antibody of low haemolytic efficiency (7S) were first recognizable after 24 hours in cultures taken from mice immunized 4 days before killing. This may represent a true genesis of new 7S-producing cells,



or it may reflect the preferential survival of cells which were masked in earlier assays by the higher proportion of 19S-producers. After 4-6 days of incubation, numbers of plaque-forming cells had dropped to low levels in all cultures. About 20% of the cells were viable at 4 days.

One attempt to culture cells from the popliteal efferent lymph of a sheep immunized with Salmonella muenchen suggested that these free-floating cells may be more resistant to in vitro conditions. Numbers of cells producing antibacterial antibody decreased only 5-fold in 7 days of culturing under the same conditions as described for mouse spleen. The active cells observed after 5-7 days of culture were about 50% smaller in average diameter than the original plaque-forming cells taken fresh from the lymph. These cells were very fragile however, and invariably disintegrated when efforts were made to smear them.

(a). Irradiation.

Adult CBA mice (usually 10-14 weeks old and of either sex) received 1400r of whole-body gamma irradiation from a <sup>60</sup>Co source, at a rate of about 14.5 rads/minute. Irradiated mice were fed on pellets containing 0.05% chloroxystin and 0.05% Achromycin. Mice

B. COLONY FORMATION IN THE SPLEENS OF IRRADIATED  
ISOLOGOUS MICE.

The use of lethally irradiated animals as immunologically inert culture chambers has been discussed in chapter 1. Kennedy et al. (1966) and Playfair et al. (1965) observed that when a relatively small number of normal spleen or lymph node cells was injected intravenously into an irradiated recipient mouse, subsequent antigenic stimulation provoked the appearance of small, anatomically localised regions of antibody-forming tissue in the spleen. Kennedy et al. estimated the average number of plaque-forming cells in these regions by parallel Jerne-type assays on whole spleens of similarly treated mice. This section describes the direct measurement of cells forming 19S and 7S haemolytic antibody in colonies of this type.

I. The recipient mice.

(a). Irradiation.

Adult CBA mice (usually 10-14 weeks old and of either sex) received 1300r of whole-body gamma irradiation from a  $^{60}\text{Co}$  source, at a rate of about 14.8 rads/minute. Irradiated mice were fed on pellets containing 0.09% chloromycetin and 0.05% Achromycin. Most

TABLE 7.1

"Background" levels of plaque-forming cells in the spleens of irradiated adult CBA mice. Each figure represents the mean number of active cells in 2 spleens. Mice received injections of bone marrow ( $2 \times 10^6$  isologous cells), or sheep erythrocytes ( $5 \times 10^8$  cells) in the combinations indicated, 1 day after 1300rads of whole body irradiation.

Bone marrow:		+	+	-	-
Antigen:		+	-	+	-
Days after injection	0				35
	3	35	15	43	13
	4	58	20	13	33
	5	6	80	10	8
	8	35	30	13	10
	11	55	3	13	35

of these animals survived for at least 12 days after irradiation without injections of bone marrow or spleen cells.

(b). Injection of donor spleen cells and antigen.

The spleen cells, together with  $5 \times 10^8$  sheep erythrocytes, were injected intravenously into recipients on the day following irradiation.

(c). Assay for plaque-forming cells.

In most of these experiments, a very small number of active cells was mixed, in the recipient tissue, with a large number of host cells. Jerne-type assays were therefore routinely used to detect the plaque-forming cells. Cells producing 19S or 7S antibody were assayed in parallel on separate plates.

(d). "Background".

Numbers of plaque-forming cells in the spleens of otherwise untreated irradiated mice were comparable to those found in unstimulated normal mice. Table 7.1 records this "background", which varied greatly between spleens but was unaffected by the injection of  $2 \times 10^6$  bone marrow cells of  $5 \times 10^8$  sheep red cells, or by the interval between irradiation and testing. Unstimulated mice, whether irradiated or not, were never found to have cells producing 7S antibody in their spleens.



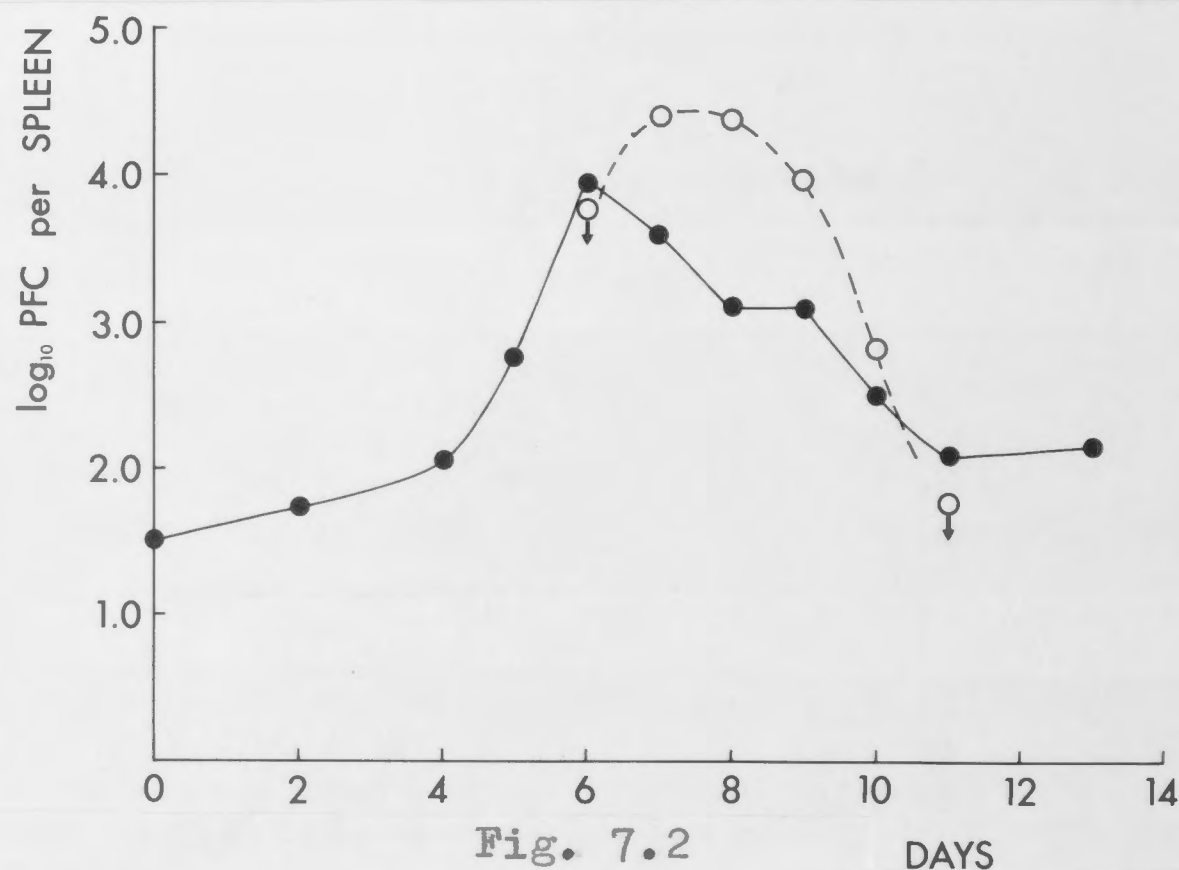


Fig. 7.2

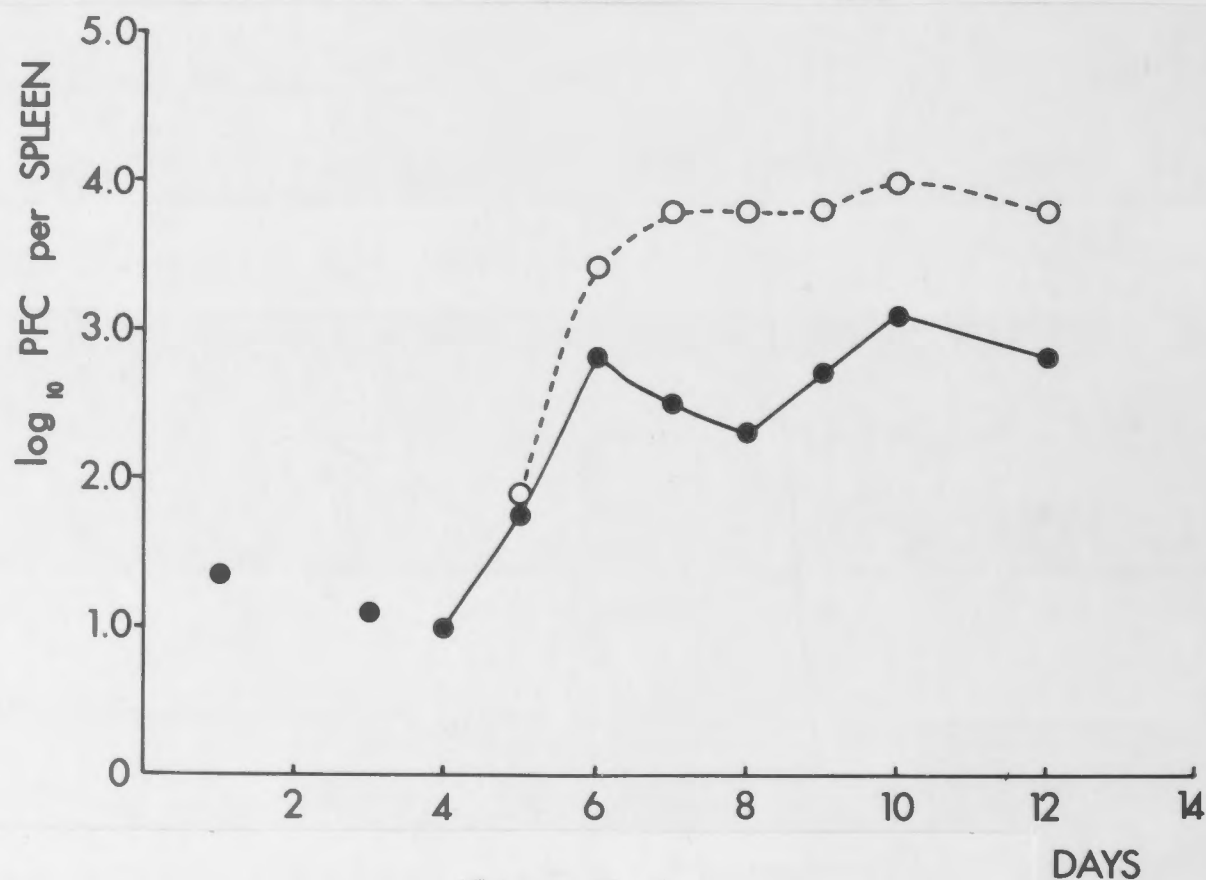


Fig. 7.3

Fig. 7.2. Antibody-forming cells in the spleens of irradiated recipient mice at intervals after intravenous injection of  $10^7$  normal mouse spleen cells plus antigen ( $5 \times 10^8$  sheep erythrocytes).

Fig. 7.3. Irradiated recipients injected with  $5 \times 10^5$  spleen cells from primed donor mice, together with  $5 \times 10^8$  sheep erythrocytes.

Unbroken lines represent numbers of cells producing 19S antibody, dashed lines follow cells releasing 7S antibody. Each point is derived from a pool of 2 recipient spleens. 95% confidence limits of each assay are less than  $0.2 \log_{10}$  units in most cases.

## II. The response of large numbers of donor cells to antigenic stimulation.

Before beginning a study of single colonies, the response of whole spleens from recipient mice was followed after injection of relatively large doses of spleen cells with antigen. Figure 7.2 shows the numbers of 19S and 7S antibody-forming cells in the spleens of such recipients after injection of  $10^7$  normal isologous living spleen cells together with  $5 \times 10^8$  sheep erythrocytes. Fig. 7.3 represents the response of  $5 \times 10^5$  primed spleen cells to the same dose of antigen; donor mice had been immunized with 2 injections of sheep red cells 3 months and  $2\frac{1}{2}$  months previously. In the primary response, cells producing 7S antibody were first seen 7 days after immunization; both 19S and 7S producers disappeared after 10-11 days. By contrast, in the secondary response, both types of cells appeared together, and large numbers could still be found in the recipient spleens after 12 days.

## III. Colonies of plaque-forming cells.

### (a). Method of assay.

It was thought that anatomically discrete clusters of plaque-forming cells could best be recognized by cutting up spleens into a large number of pieces, then

assaying each piece separately for active cells. However, when preliminary attempts to subdivide spleens were observed under a low magnification, it was found that the act of cutting a section of the spleen liberated large numbers of single cells and clumps of cells which then contaminated subsequent sections. The procedure finally adopted was as follows:-

Sufficient normal spleen cells ( $1.4 \times 10^6$  or  $2 \times 10^6$  living nucleated cells) were injected intravenously with antigen into irradiated mice to produce an average of less than 1 colony per recipient spleen. Recipient mice were sacrificed at intervals, and their spleens were dried on filter paper and cut into 8 approximately equal slices with a sharp razor blade, a clean blade being used for each cut. A suspension of dispersed cells was then prepared from each slice by gentle rubbing on a small piece of 400-mesh wire. Each batch of cells was assayed in parallel on 2 duplicate Jerne plates. When the whole spleen had been divided up in this way, the 16 plates were incubated for 1 hour at  $37^{\circ}\text{C}$ . One ml of anti-7S antiserum, at a dilution of 1/200, was then pipetted on to one of each pair of duplicate plates. After a further hour of incubation, this was poured off, and 1ml 1/10

TABLE 7.2

Colonies of plaque-forming cells in the segments of spleens from irradiated mice at various times after injection with normal spleen cells and sheep erythrocytes. Column A represents numbers of cells releasing 19S antibody, and Column B, plaque counts on plates to which antiglobulin was added before complement. Colonies are marked with an asterisk: high numbers of plaque-forming cells in adjacent segments are considered to belong to the same colony (see text).

Spleen no.		1		2		3		4	
Days after antigen stimulation.		6		7		8		8	
		A	B	A	B	A	B	A	B
Segment no.	1	0	0	3	1	11	5	2	1
	2	2	0	1	1	1	5	5	4
	3	0	1	6	6	5	10	4	5
	4	2	1	2	0	57*	700*	7	3
	5	2	1	1	2	11*	210*	9	4
	6	1	0	6	2	4	1	7	0
	7	1	0	16*	9	3	10	4	35*
	8	0	0	14*	5	9	10	3	21*

Spleen no.		5		6		7		8	
Days after antigen stimulation.		9		9		10		12	
		A	B	A	B	A	B	A	B
Segment no.	1	1	3	4	0	101*	79	48*	135*
	2	3	2	10	1	30	18	11*	22*
	3	6	4	13	3	6	9	6	6
	4	3	0	6	3	40*	20	20*	14
	5	4	0	23	18	75*	22	2	1
	6	3	120*	11	3	30	18	5	3
	7	3	140*	17	2	30	20	1	2
	8	1	0	15	4	19	7	10	13



complement was added to all plates for 30 minutes' final incubation before counting plaques.

(b). Criteria for colonies.

Table 7.2 shows the plaque counts from 8 spleens, chosen to indicate the variety of responses obtained. Column A represents the assay for plaques due to 19S antibody, and column B, the numbers of plaques counted on plates to which antiglobulin had been added. Two main problems were encountered in identifying colonies. First, the "background" of 19S plaques varied considerably between spleens; number 1 had a low background (table 7.2), spleen 2 an average level. Second, it can be seen that antiglobulin serum added to the B plates usually depressed the number of 19S plaques to roughly half the number counted on the corresponding A plates.

(c). 19S colonies.

A segment was considered to harbour a 19S colony if at least 10 plaques were counted on the plate, and if this count was more than 3 times higher than average background levels in other segments. Where counts were high in 2 adjacent segments, both groups of cells were scored as belonging to the same colony. The actual number of plaque-forming cells in the colony was determined by first subtracting the average

background level found over the rest of the spleen, then multiplying the remaining figure by 2 to allow for the fact that only half the spleen segment was assayed on the "19S" plate. Spleen 2 thus contained 1 colony of about 48 cells releasing 19S antibody.

(d). 7S colonies.

A segment was scored as containing 7S-producing cells if the B plate contained significantly more plaques than the corresponding A plate. From such a B plate with  $y$  plaques, the total number of cells in the segment releasing 7S antibody was calculated as  $2(y - \frac{x}{2})$ , where  $x$  was the count on the A plate. In spleen 5, a clearly demarcated 7S colony may be seen.

In spleen 3, a 19S and a 7S colony were closely associated. Segment A4 of this spleen satisfied the criteria for a 19S colony, with perhaps a few cells from the colony in segment A5, where the count was about twice background. The total size of this colony was estimated at 116 cells. Cells from segments B4 and B5 together produced 910 plaques; the size of the 7S colony was estimated as  $2(910 - 58)$  or approximately 1700 cells.

These criteria for identification of colonies are stringent - small colonies would not be recognized. A group of cells producing 7S antibody would have to

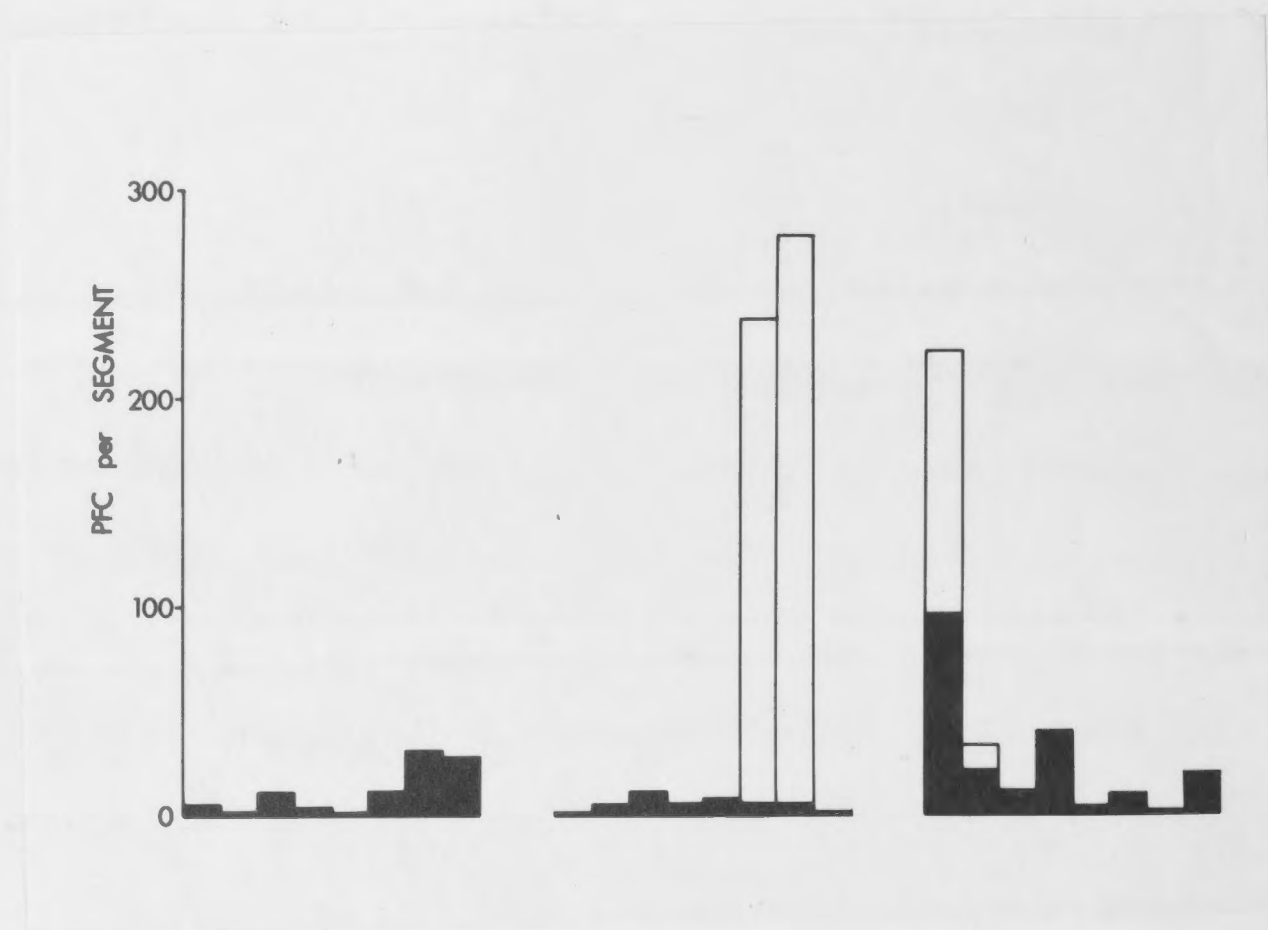


Fig. 7.4

Plaque-forming cells in the 8 segments of 3 spleens from irradiated mice injected with normal spleen cells plus sheep erythrocytes (spleens 2, 5 and 8 from table 7.2 are shown. Black columns: 19S-producing cells. Where 7S-producing cells were identified their numbers are represented by the height of the white columns.

be about twice as numerous as the 19S-producers from the same segment in order to be classified as a colony. This stringency was necessary because of the relatively high and variable background encountered in these spleens. Fig. 7.4 shows colonies from 3 spleens in histogram form.

(e). Background.

Numbers of background plaques tended to increase with time after immunization. There was also a marked tendency for colonies to appear in those spleens where background was high. Mice which received antigen but no spleen cells did not develop increased numbers of plaques (table 7.1). Assays of spleens from animals injected with spleen cells and no antigen are needed to decide whether this increased background is due to antigenic stimulation of donor cells; it may be that plaque-forming cells tend to leak away from colonies. One spleen assayed 1 day after injection of normal spleen cells plus antigen contained a distinct colony of about 80 plaque-forming cells; this may have been of host origin.

(f). Kinetics of colony development.

Figure 7.5 shows the results of 54 spleen assays. 31 colonies were identified, 22 of 19S-producing cells, and 9 of 7S-producers. The pattern of appearance and



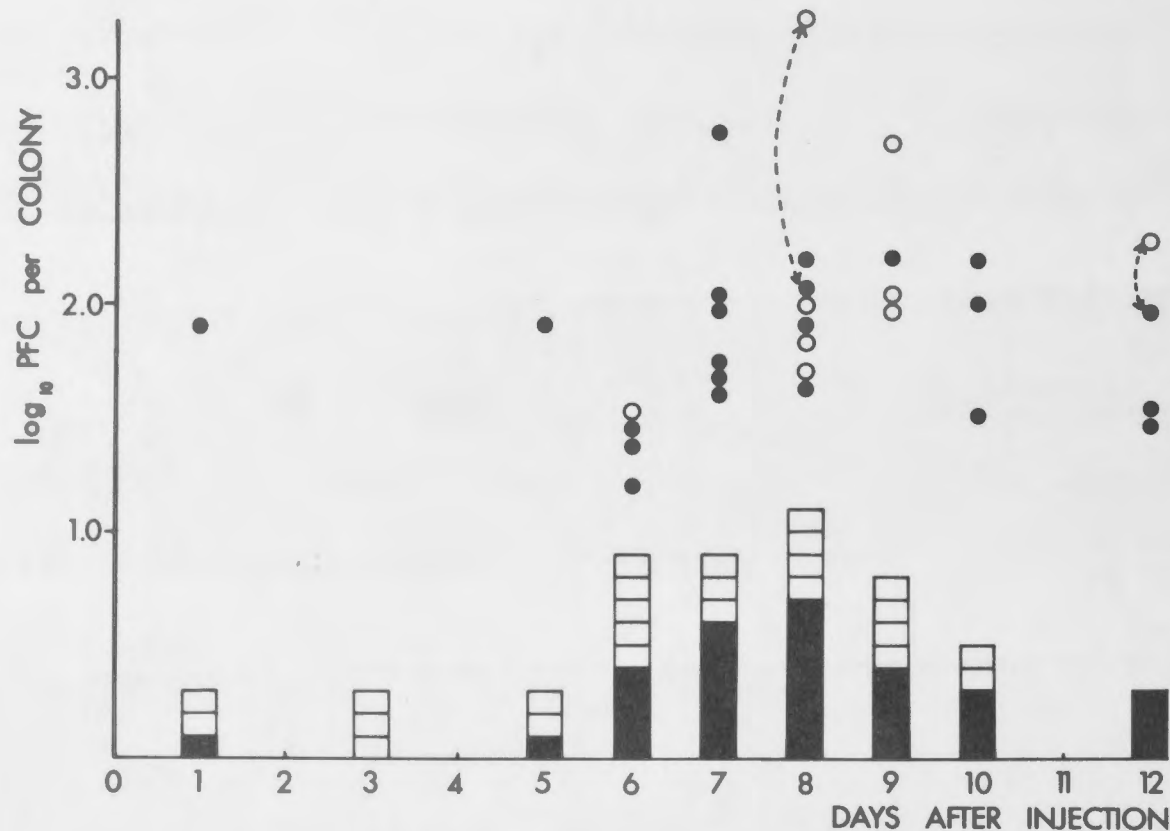


Fig. 7.5

Development of colonies of antibody-forming cells in irradiated spleens. Each circle represents 1 colony. Black circles: numbers of cells producing 19S antibody in a colony. Open circles: 7S-producing cells. Two closely associated 19S and 7S colonies were found (see text), and the numbers of cells producing 19S or 7S antibody in these "double" colonies are connected by dashed lines.

The lower histograms compare the number of colonies found (black columns) with the total number of spleens examined (total height of columns). At 12 days after immunization, the 3 spleens studied contained a total of 4 colonies.

growth of colonies is roughly that expected from earlier experiments where a relatively large inoculum of cells and whole spleen assays were used. Colonies varied greatly in size, suggesting that most of the antibody-formers in the spleens of mice which received large numbers of cells may have belonged to 1 or 2 large colonies.

(g) Association of 19S and 7S colonies.

Two of the 9 7S colonies occurred in the same segments as 19S colonies. (table 7.2, spleens 3 and 8). Assuming that each colony can be focussed anywhere within a segment, such an association of 2 out of 9 7S colonies would be expected about 8% of the time by chance (appendix 1.4). However, in both these "doubles", the proportion of 7S to 19S plaque-forming cells is roughly the same over 2 segments; this suggests that the colonies in fact overlap completely, and that they would have been found to coincide if spleens had been cut into, say, 100 pieces instead of 8.

A significant tendency for association between colonies of the 2 types could be explained in 2 ways; the 7S colonies arise separately, from different precursor cells, but develop best when closely associated with 19S producing cells; or 7S-producing cells arise from 19S-producing cells, as suggested by

Nossal et al. (1964). The latter explanation seems much the more probable one. 19S production may well cease shortly after 7S production begins, leaving a colony with only plaque-forming cells of the second type.

#### IV. Morphology of cells from single colonies.

For the work described in the previous section, each spleen segment was reduced to a suspension of dispersed cells, all of which was then used in assaying for plaque-forming cells. If only a proportion of each suspension were used for such assays, the remainder could be kept at 0°C until the segments containing colonies were identified; the other part of these colonies would then be available for study. If colonies are indeed clones, it would be of great interest to compare the morphology of different members of the clone at the same time after antigenic stimulation of the precursor cell. A preliminary experiment reported here indicated that such a study would be technically feasible.

Two colonies, each comprising about 100 cells releasing 19S antibody, were obtained from the spleens of 2 recipient mice 7 days after injection of normal spleen cells and antigen. The 2-step process described in chapter 3 was used to isolate 4 plaque-forming cells

TABLE 7.3

Morphology of cells from single colonies.

Colony no.	Cell no.	Average diam ( $\mu$ )	Basophilia	Ratio nucleus/ cytoplasm
1	1	13	$2\frac{1}{2}$	0.4
	2	12	$2\frac{1}{2}$	0.6
	3	12	$2\frac{1}{2}$	0.4
	4	?	$2\frac{1}{2}$	in mitosis.
2	1	14	2	0.5
	2	15	2	0.6
	3	15	2	0.5
	4	16	2	0.5
	5	12	3	0.5
	6	12	3	0.6



from the first colony and 6 from the second. These individual cells were smeared and stained with Leishman's stain. In table 7.3 morphological features of each cell are recorded. All 4 plaque-forming cells from the first colony looked the same, except that one was in mitosis. In the second group, 4 cells were closely similar, and the remaining 2 were quite different although they closely resembled one another. The similarities between cells were much more striking than the figures in table 7.3 indicate; within each of the 3 groups, each member had the same degree of apparent "granularity" of nucleus and cytoplasm, and stained to exactly the same shade.

"silent" or non-functional viruses could be identified. In the experiments described in this paper, a small number of clones may well have been identified, while most of the cells were from a single clone. A method is needed for the rapid identification of multiplying *in vitro*. Another method is the use of colonies of bone marrow cells in a semi-solid medium, a feeder layer of kidney cells; this technique might be applicable to the anti-hepatitis system.

The importance of the *in vitro* colony method of Kennedy and of Playfair is that the colonies which they describe may be single clones. Both groups have

### C. DISCUSSION.

The brief attempts at in vitro culture of antibody-forming cells reported here were relatively unsuccessful. Many workers have found that the test-tube is a poor substitute for the intact animal when differentiated cells are to be cultured. However, in vitro culture has one great potential advantage over in vivo work; the progeny of a single cell or population of cells can be localised. In effect, an extra marker, position, is available. If a single clone could be grown in vitro, there would be no need to rely on a functional test (antibody release) to detect members of the clone, and "silent" or non-functional members could be identified. In the experiments described in this chapter, a small number of clones may well have been multiplying normally while most of the cells were dedifferentiating or dying. A method is needed for the rapid detection of clones multiplying in vitro. Bradley and Metcalf (1966) grew colonies of bone marrow cells in sloppy agar over a feeder layer of kidney cells; this technique might be applicable to the antibody-forming system.

The importance of the in vivo colony method of Kennedy and of Playfair is that the colonies which they describe may be single clones. Both groups have

adduced statistical support for this idea. However, it is known that a "one-hit" dose-response curve can be obtained if an event (colony formation) is due to the combined action of 1 single unit from the input population and a relatively large number of assisting units (Coppleson and Michie, 1966). It is of crucial importance to establish that the colonies of antibody-forming cells are in fact clones, and a direct demonstration should be attempted. One method of proof would involve tritium labelling of the antigen-sensitive precursor cells which are thought to give rise to colonies, perhaps by a prolonged course of injections administered to a donor mouse. Cells from this labelled mouse would now be mixed with an equal number of cells from an unlabelled mouse, and the mixture injected into irradiated recipients. If colonies arise from single cells, one would expect to find consistently that either all or none of the antibody-forming cells in any 1 colony were labelled. Mixed labelling would indicate that a colony originated from more than 1 precursor cell.

The chief advantage of in vivo cloning methods over any in vitro counterpart which might be developed is that while conditions in the irradiated recipient are likely to resemble the normal host environment, there is less guarantee that the behaviour of cells

in vitro will reflect their role in vivo. The main disadvantage of the host spleen, as pointed out earlier, is that only those cells which are releasing haemolytic antibody can be isolated from the vast mass of irrelevant host tissue.

Two new properties of spleen colonies have been discovered using the method described in this chapter. First, direct assay of the plaque-forming cells from colonies has demonstrated a striking heterogeneity in colony size. This information is lost when average sizes are estimated by the method of Kennedy et al. Secondly, colonies of cells releasing 7S antibody were identified. These may arise from cells producing 19S antibody, as suggested by Nossal. To prove this in the present system, more assays at a lower colony multiplicity are required; if 19S and 7S colonies are really associated at some stage of their growth, the proportion of such associations should not change as the overall incidence of colonies decreases, while the probability of associations due to chance would drop sharply.

The average size and kinetics of growth of 19S colonies observed in the present study agree well with the data of Kennedy's group. The incidence of antigen-sensitive precursors in unprimed normal spleens appeared



to be about 1 per 2 million cells, or about 2-4 times lower than the value obtained by Kennedy et al. and by Playfair's group. This difference may be due to the obscuring of small colonies by "background" plaques. Kennedy states that, in their C57/Bl mice, background seldom exceeded 10 plaque-forming cells in any individual spleen, a surprising observation in view of the much higher levels reported by Wigzell et al. (1965) in an extensive survey of the incidence of plaque-forming cells in the spleens of normal mice of various strains. Colony formation has not yet been followed using donor spleens from primed mice; one preliminary test suggested that the incidence of antigen-sensitive precursors was about 100 time higher than in the spleens of unprimed mice.

If it can be proved that the spleen colonies are in fact single clones, then a very powerful model system has been found for studying cell differentiation. Morphological examination of isotope-labelled cells at various stages of clonal development would provide, for the first time, direct information on the developmental sequences followed by the progeny of one precursor cell. The proteins produced by different members of the same clone could also be compared; it would be relatively easy to detect heterogeneity of

antibody by testing plaque-forming cells against mixed monolayers of sheep and cross-reacting (goat, cow) erythrocytes, or using bacterial lipopolysaccharide antigens, as described in chapter 5. More optimistically, if it is true that a single cell "switches" from 19S to 7S antibody production during development, a means of studying the factors which influence this most interesting differentiative event may be at hand.

by Jarne-type assays of all, or part of, dispersed cell preparations from each segment: a fraction of the cells from each segment could be kept for subsequent study after colonies had been identified.

Two new findings are reported. First, there is a more than 20-fold variation in the size of different colonies on the same day after antigenic stimulation. Secondly, cells releasing antibody of low haemagglutinating efficiency (7S) also appear in colonies during the primary response of lymph cells in irradiated spleens. These seem to be associated with 19S colonies more often than would be expected by chance, suggesting that the cells producing 7S antibody develop from 19S producers.

D. SUMMARY.

Some relatively unproductive attempts to culture antibody-forming cells in vitro are described.

The spleen colony model of Kennedy et al. (1966) and Playfair et al. (1965) has been investigated. In the present study, the spleens of recipient mice were cut into 8 segments, and colonies were detected by Jerne-type assays of all, or part of, dispersed cell preparations from each segment; a fraction of the cells from each segment could be kept for subsequent study after colonies had been identified.

Two new findings are reported. First, there is a more than 20-fold variation in the size of different colonies on the same day after antigenic stimulation. Secondly, cells releasing antibody of low haemolytic efficiency (7S) also appear in colonies during the primary response of donor cells in irradiated spleens. These seem to be associated with 19S colonies more often than would be expected by chance, suggesting that the cells producing 7S antibody develop from 19S producers.

In chapter 1, 3 properties of the precursor cell of the antibody-forming series were discussed: morphology, synthesis of DNA, and range of potential reactivity. This chapter briefly describes experiments aimed at deciding whether the precursor cell is a dividing or non-dividing type. Also, experimental methods are proposed for determining the morphology and range of reactivity of these cells.

## CHAPTER 8

### A. DNA SYNTHESIS OF PRECURSOR CELLS.

#### 1. Method.

#### THE PRECURSOR CELL.

Donor adult mice were given intraperitoneal injections of 100µc of tritiated thymidine in 0.1ml (1mc/ml, 16mc/mM; Radiochemical Centre, Amersham, England). Injection times were approximately 26, 14, and 1-2 hours before killing the mouse. At sacrifice the spleen was removed, and a dispersed cell suspension made. These cells were washed twice at 240g in Eagle's medium, and the number able to exclude trypan blue estimated. From  $3-24 \times 10^6$  of these cells, plus  $5 \times 10^8$  sheep red cells were then injected intravenously into each of 2 recipient mice which had been subjected to 1300rads of total body gamma irradiation 24 hours before. Recipient mice were sacrificed at 3, 4, 5 or 6 days



In chapter 1, 3 properties of the precursor cell of the antibody-forming series were discussed; morphology, synthesis of DNA, and range of potential reactivity. This chapter briefly describes experiments aimed at deciding whether the precursor cell is a dividing or non-dividing type. Also, experimental methods are proposed for determining the morphology and range of reactivity of these cells.

#### A. DNA SYNTHESIS BY PRECURSOR CELLS.

##### I. Method.

Donor adult CBA mice were given 3 intraperitoneal injections of 100 $\mu$ c of tritiated thymidine in 0.1ml (1mc/ml, 16mc/mM; Radiochemical Centre, Amersham, England). Injection times were approximately 26, 14, and 1-2 hours before killing the mouse. At sacrifice the spleen was removed, and a dispersed cell suspension made. These cells were washed twice at 240g in Eagle's medium, and the number able to exclude trypan blue estimated. From 3-24x10<sup>6</sup> of these cells, plus 5x10<sup>8</sup> sheep red cells were then injected intravenously into each of 2 recipient mice which had been subjected to 1300rads of total body gamma irradiation 24 hours before.

Recipient mice were sacrificed at 3,4,5 or 6 days

after receiving the donor cells and antigen. By this time plaque-forming cells, presumably of donor origin, could be found in suspensions of cells from recipients' spleens. Single active cells were picked out as described in chapter 3, and smeared on gelatin-coated slides. Usually 5-10 such cells were smeared on one slide, which was then fixed in methanol for 1 hour. The cells were located by low power phase contrast scanning, and their positions marked. Stripping film (Kodak AR 10) was applied to the slides, which were exposed for 21 days. After developing and fixing this film, the cells were lightly stained with Azur A, and examined for grains.

This experimental design is a modification of that used by Nossal and Makela (1962b). By transferring a limited population of prelabelled cells to an irradiated host before antigenic stimulation, it was hoped that significant reutilization of label could be avoided. The 3 injections of tritiated thymidine given to the donor mouse were intended to label most rapidly-dividing cells; from 2-5% of this population of spleen cells incorporated the isotope. Many of these cells were very heavily labelled and it is quite possible that some cells were prevented from further division or killed by the large doses of tritiated

thymidine used. However, the capacity of the transferred spleen population to mediate an adoptive antibody response was unimpaired.  $2 \times 10^7$  injected donor spleen cells thus contained not more than  $10^6$  labelled cells, of which about 10-20% would be expected to reach the spleen (Siminovitch et al. 1963). The quantity of labelled cellular DNA available for reutilization in the spleen was therefore very small. Control smears of large numbers of the cells from spleens of recipient mice were always subjected to autoradiography in parallel with the plaque-forming cells, and it was found that labelled cells were very rare among them (about 1/3000).

For studies on the precursor cell of the secondary response, donor mice were primed by intravenous injection of tritiated thymidine. Spleens of recipient mice were assayed for antibody-forming cells in the same way except that antiglobulin was added to the medium to enable detection of 19S or 7S-producing cells.

Significant numbers of plaque-forming cells usually appeared in the spleens of recipient mice 4 days after injection of donor cells plus antigen (see figs. 7.2 and 7.3), or 3 days after transfer if the donor had been primed. It was obviously desirable to study plaque-forming cells as soon as possible after antigenic

stimulation in order to avoid the loss, by repeated division, of any radioisotope which they might have contained (discussed below). On the other hand, active cells were technically much more difficult to isolate when rare in the recipient spleens, and in addition, at such times "background" plaque-forming cells of recipient origin could be expected to be encountered relatively often. In practice, antibody-formers were only taken from spleens containing at least 100 active cells. In any one experiment, cells were usually obtained from 2 or 3 recipients sacrificed on consecutive days.

Early experiments differed slightly in design from the above. Individual antibody-forming cells were stained with Leishman's, examined, then decolorized for 24 hours in 4 changes of methanol before applying stripping film. This procedure led to some formation of non-specific grains above isolated cells, (due to persisting stain?). Numbers of grains above plaque-forming cells were compared with numbers above cells in the control smears of the recipient's whole spleen population. Control counts were as high as 2-5 grains per cell, and on occasion more than 10 grains per cell in these pre-stained preparations, whereas in control smears which had not been prestained with Leishman's the level was usually much lower than 1 grain/cell.



TABLE 8.1

Expt. no.	Primary or secondary	Days after injection that recipient killed.	Plaque- forming cells per spleen	Cells examined	
				Unlabelled	Labelled (no. of grains).
1	primary	4	100	2	0
		5	2000	21	0
2	secondary	3	100	1	0
		4	2000	21	0
		5	100000	26	1(17or11)*
3	primary	4	300	6	0
		5	5000	6	0
4	secondary	4	600	3	0
		5	50000	17	1(6)

\* This was a pair of cells, joined together, which formed 1 plaque. 1 cell had 17 grains over it, the other 11.

## II. Results.

### (a). Criteria adopted for positive labelling.

A cell was not considered to be labelled if:

- (i) it had less than 3 grains over, or within  $1\mu$  of, the nucleus.
- (ii) it had less than 5 times as many grains as an average of adjacent background areas.
- (iii) "background" averaged more than  $2 \text{ grains}/150\mu^2$ .

(If background levels were higher than this, the cells were not scored at all).

### (b). Cells not stained before applying film.

Table 8.1 shows that of 105 antibody-forming cells examined so far, only 2 have been labelled. "One" of these was a pair of joined cells, which together formed 1 plaque. All other figures are for single cells.

### (c). Cells prestained with Leishman's, then decolorized.

Table 8.2 shows the much higher, presumably non-specific grain counts obtained by this procedure. The 2 cells which had approximately 70 and 200 grains over their nuclei obviously do not belong, in a statistical sense, to the main population, and probably contained radioisotope. A small number of genuinely labelled cells could have passed undetected in this population.

TABLE 8.2

Number of grains over antibody-forming cells  
which had been prestained with Leishman's, then  
decolorized, before applying stripping film.

	No. of grains	No. of cells in class.
	0-4	52
	5-9	15
	10-14	6
	15-19	6
	20-24	2
	25-69	0
	70	1
approx.	200	1

### III. Discussion.

The results indicate that antigen-sensitive precursor cells, in either primary or secondary responses, are not a continuously dividing cell line. Against this tentative conclusion it may be argued that sufficient cell division took place to dilute all label from the active cells. Injection of  $2 \times 10^7$  unprimed cells into a recipient mouse should give rise to about 20 colonies in the spleen (chapter 7), so that the 100 cells in the spleen 4 days later might have arisen by an average of 3 division per precursor cell. On the other hand, most of these 100 cells could belong to 1 or 2 more rapidly growing clones, in which case the chances of loss of label by dilution would be greater. Two controls are proposed to test the importance of this effect.

(i). Administering tritiated thymidine daily to donor mice for periods of 1 month or more before sacrifice. An increase in the proportion of labelled antibody-forming cells in recipients' spleens would demonstrate the significance of the negative results obtained so far.

(ii). Injecting antigen into the donor mouse, followed by 3 spaced doses of tritiated thymidine, then transferring donor cells without antigen to recipients and



examining antibody-forming cells at various times to determine the time needed for total loss of label by division.

#### B. MORPHOLOGY OF THE PRECURSOR CELL.

In the work of Gowans and McGregor (1963) already referred to, the ability to produce antibody was fully restored to X-irradiated rats by an injection of thoracic duct lymphocytes. More than 99% of the rescuing inoculum were small lymphocytes, which strongly suggests that these cells eventually generate antibody-forming progeny.

In such experiments it is always possible that a very small number of contaminating cells of another morphological type may have been responsible for subsequent antibody formation. Even when most of the larger cells of thoracic duct lymph are removed by prior incubation, it could be argued that the remaining contaminants multiplied sufficiently to make up their numbers again. This possibility could be minimised by assaying the population of donor cells for colony-forming precursors rather than by examining the sera of recipients for antibody. One might attempt to purify the small lymphocytes in several different ways,

for example by density gradient centrifugation and on glass bead columns (Shortman, 1966), then test each of the purified populations directly for its content of cells capable of giving rise to colonies.

C. THE NUMBER OF ANTIGENS TO WHICH A SINGLE  
IMMUNOLOGICALLY COMPETENT CELL CAN REACT:

ANTIGEN COMPETITION.

The simplest way to determine the range of competence of a single cell would be to test it with a randomly selected antigen. If it responded, the chances would be high that it could have responded to any such antigen. Essentially, this is what the colony-transfer experiments discussed in chapter 1 tried to do. They showed that an irradiated animal's immunological competence could be fully restored with what was believed to be a single clone of cells. The objections to this experimental design were discussed. Firstly, the haemopoietic nodules transferred were almost certainly not pure clones. Secondly, considerable time elapsed between the early growth of the clone and the testing of its immunological competence. Obviously, a method is needed which will analyse the

ability of an antigen-sensitive progenitor cell to react at the moment of testing.

The phenomenon of antigenic competition (reviewed by Adler, 1964), at first sight suggests that precursor cells are multipotential. The observation is that if 2 antigens, A and B are simultaneously injected into an animal, in some situations only half as much antibody to A is produced as would have appeared if B had not been injected. If anti-A and anti-B precursor cells were committed, one would have expected the 2 responses to be independent. There are 2 main difficulties in the path of the conclusion that antigen competition represents direct competition for multipotential precursor cells. The first is that the amount of antibody in the serum reflects only distantly the number of precursor cells stimulated. The second is that the competition "block" may occur at some point other than the precursor cell; perhaps at the macrophage level. A macrophage saturated with A might not act as a processing agent for B.

The first of these objections could probably be eliminated by assaying not for antibody, or for antibody-forming cells, but directly for the precursor cells themselves. Antigen competition could be tested for in mice restored with, say  $10^6$  isologous spleen cells,

then challenged with mixtures of erythrocytes. Spleens would later be examined for colonies of plaque-forming cells. The demonstration of a reciprocal drop in anti-A and anti-B colonies when both antigens were injected together would be a prima facie case for competition for some of the cells in the donor population of spleen cells. Conversely, an additive response would suggest committedness on the part of these cells.

To demonstrate the point during induction at which a competition for antigens occurred, it would be necessary to break the process down into stages. The 2-step induction method of Ford, Gowans and McCulloch (1966) might be used; an in vivo priming of macrophages with antigen, followed by an in vitro incubation with thoracic duct cells which are then allowed to multiply and produce antibody in an irradiated recipient. The experiment would be; (i) prime 2 populations of macrophages separately with antigens A and B; (ii) compare the effect of incubating a standard lymphoid cell population with either 1 or both of the primed macrophage populations. "Induced" lymphoid cells would be assayed by colony formation in the spleens of irradiated hosts. If twice as many colonies could be initiated by exposing lymphoid cells to both lots of primed macrophages



together, this would suggest committedness in the lymphoid cells. Competition might then be demonstrated within macrophages by treating 1 population with both antigens together before incubating them with the lymphoid cells.

Mice were labelled with  $^3$  intravenously by injection of tritiated thymidine at 12-hourly intervals. 1-2 hours after the last injection, washed cells from this animal were injected into irradiated isologous recipients, together with sheep erythrocytes as antigen. The antibody-forming cells which appeared in spleens of recipients several days later were examined autoradiographically for evidence of DNA synthesis by precursor cells before antigenic stimulation. The great majority of the antibody-forming cells examined so far have been unlabelled, suggesting that the precursor cells did not incorporate tritiated thymidine. However, the significance of these negative results will need to be tested by 2 controls, which are discussed. The use of a cell-transfer system appears to have eliminated significant reutilization of label.

Methods are proposed for determining the morphology and range of antibody-forming potential of precursor cells.

D. SUMMARY.

An attempt has been made to determine whether the antigen-sensitive progenitor of antibody-forming cells is a dividing or non-dividing cell type. Donor mice were labelled with 3 intravenous injections of tritiated thymidine at 12-hourly intervals. 1-2 hours after the last injection, washed spleen cells from this animal were injected into irradiated isologous recipients, together with sheep erythrocytes as antigen. The antibody-forming cells which appeared in spleens of recipients several days later were examined autoradiographically for evidence of DNA synthesis by precursor cells before antigenic stimulation. The great majority of the antibody-forming cells examined so far have been unlabelled, suggesting that the precursor cells did not incorporate tritiated thymidine. However, the significance of these negative results will need to be tested by 2 controls, which are discussed. The use of a cell-transfer system appears to have eliminated significant reutilization of label.

Methods are proposed for determining the morphology and range of antibody-forming potential of precursor cells.

From the intense cellular activity which follows antigenic stimulation of an animal, a new protein, antibody, emerges. This process has been traditionally monitored by titrating serum for antibody activity. The Jerne-Ingraham plaque technique has replaced the assay of antibody-forming cells in the field of simplicity as a serum analyzer, and also provides a much more direct measure of antibody activity than serum titration can give.

A modification of the plaque technique has been described in Chapter 2. This has 2

## CHAPTER 9

advantages over the traditional method; it is more sensitive, and permits the study of the living

## CONCLUSIONS.

plaque-forming cells of the living animal. To achieve this, good utilization of the growing power of the "free suspension" cell line, which has been used for modification tests about 100 times less cells per activity than the agar plaque technique.

Just as plaque formation can be used to detect small numbers of cells producing virus, so it may be employed in a screen for the virus releasing antibodies of 2 defined specificities. It was shown in Chapter 5 that immunizing mice with 2 antigens on the same individual erythrocyte did not give rise to detectable numbers of cells producing both corresponding

From the intense cellular activity which follows antigenic stimulation of an animal, a new protein, antibody, emerges. This process has been traditionally monitored by titrating serum for antibody activity. The Jerne-Ingraham plaque technique has reduced the assay of antibody-forming cells to the same level of simplicity as a serum titration, and its application allows a much more direct measure of cellular events than serum titrations can ever provide.

A modification of the plaque technique has been described and standardized in chapter 2. This has 2 main advantages over the original; it is more sensitive, and perhaps more important, it allows the living plaque-forming cells to be clearly seen. To achieve this, some sacrifice of the great screening power of the Jerne method was required, and the "free suspension" modification tests about 100 times less cells for activity than the agar plaque technique.

Just as plaque formation may be used to detect small numbers of cells producing one antibody, so it may be employed in a search for the rare cells releasing antibodies of 2 defined specificities. It was shown in chapter 5 that immunizing mice with 2 antigens on the same individual erythrocytes did not give rise to detectable numbers of cells producing both corresponding



antibodies. It is clear that the process of immunizing an animal with complex antigens, then searching for cells producing an arbitrarily selected 2 antibody specificities, can provide only a minimum estimate of the range of different antibodies which single cells can manufacture. A more positive approach to this problem, based on immunizations with defined antigens of increasing size, has been suggested.

The modified plaque technique involves incubation of monolayers of lymphoid cells and erythrocytes without any supporting medium. Such monolayers are quite stable in small drops under oil, and plaque-forming cells can be easily manipulated out of these drops and smeared individually on slides for light microscopy. Alternatively, a number of active cells can be clumped together with antiserum, to provide a specimen large enough to be readily stained and embedded for electron microscopic examination.

These new techniques were applied to a study of the antibody-forming cells in 2 model systems. The first of these was Morris's sheep lymph node preparation (Hall and Morris, 1962). It was found that a very high proportion (up to 5%) of the cells in the efferent lymph from the popliteal node of immunized sheep could release antibody to bacterial lipopolysaccharides. Most of these cells produced very small plaques which were

undetectable by the original Jerne technique. By light microscopy, the active efferent lymph cells were large and basophilic, and in the electron microscope they were found to have very small amounts of endoplasmic reticulum. While this work was in progress, the papers of de Petris et al. (1965) and Hummeler et al. (1966) were published, confirming that cells with apparently little organization for protein synthesis could contain and release antibody. Cells of this type were also found in sheep lymph nodes, but here an additional population of much smaller antibody-forming cells was identified; many of these were mature plasma cells with considerable amounts of endoplasmic reticulum.

The second model system studied was the popliteal lymph node of mice immunized with sheep erythrocytes. Cells releasing 19S or 7S haemolytic antibody were examined by light microscopy throughout the primary and secondary immune response. Antibody-forming cells exhibited considerable morphological heterogeneity. Most were basophilic mononuclears from 8-14 $\mu$  in average diameter, with a relatively large nucleus, while a smaller proportion were typical mature plasma cells. These findings are thought to explain the existing state of disagreement about the nature of the antibody-

forming cell. The larger basophilic mononuclear cells probably correspond to the immature plasma cells of Nossal (1959), Fagréus (1948), and others. The smaller basophilic mononuclears would be classified as small lymphocytes in preparations where their basophilia was not apparent. This would account for the observation of Attardi et al. (1964) that most of the cells releasing anti-phage antibody in rabbits were small lymphocytes, as judged by microscopic examination of living unstained cells. Similarly the fluorescing "small lymphocytes" of Vasquez (1961), Baney et al. (1962), van Furth <sup>et al.</sup> (1966b), and others are probably equivalent to the small basophilic mononuclears of mice. The antibody-forming cells which Hummeler et al. (1966) and Harris et al. (1966) have called small lymphocytes were identified by electron microscopic examination, and were shown to have more protein-synthesizing apparatus than the majority of their inactive fellows; in Leishman-stained smears these active cells would no doubt have exhibited a greater degree of basophilia than most small lymphocytes. Some apparently "normal", non-basophilic small lymphocytes were found to be capable of antibody production in the present study, and these were particularly common amongst a small

colonies are especially variable in size, and they

sample of plaque-forming cells from the spleens of unimmunized mice.

What to call these cells - "small lymphocytes", basophilic mononuclears, or aberrant plasma cells - may seem to be a semantic argument, and therefore unimportant. However, the point at issue is really, how are these different kinds of antibody-forming cell related to one another? Do they arise by differentiation from separate precursors, or are they all different stages in the one developmental sequence? A review of the literature shows that while a great deal of work has been done on the life history of antibody-forming cells, the enormous complexity of cellular events in stimulated lymphoid tissue has prevented the emergence of any really clear understanding of this process. Evidently, new methods for following the development of single clones are required. The recently described techniques of Kennedy et al. (1966), and Playfair et al. (1965) may be suitable, if it can be proved that the colonies they detect are, as seems most likely, single clones. In chapter 7 of this thesis, methods were defined for studying the morphology of individual members of these colonies and the antibody they produce. It was shown that the colonies are enormously variable in size, and that



they may involve cells producing 19S or 7S antibody.

Finally, in chapter 8, methods for determining some of the properties of the antigen-sensitive precursor cell are discussed. Some findings are presented which suggest that these cells are not continuously dividing, but the experiments are inconclusive as they stand, and further controls are proposed.

Interest in antibody formation transcends immunology. The process demonstrates the essential features of differentiation, and as an experimental model, has the advantage that the stimulus may be chemically defined. Because of the great activity of antibody, the product of single cells can be studied, and because of its characteristic specificity of action, populations of cells which are morphologically indistinguishable may be functionally subdivided. Study of cellular events in antibody formation may have quite broad biological implications. Further progress seems likely to depend on the development and application of techniques which can directly demonstrate the life histories of single clones.

APPENDIX 1 : Statistics.

1.1. The proportion of cells in a chamber which produce plaques.

The area of the high-power field used to count lymphoid cells throughout this work was  $0.074\text{mm}^2$ . If the area of a chamber is  $A\text{mm}^2$ , and the average number of nucleated cells in a high-power field is  $H$ , then total number of nucleated cells in the chamber

$$= H \frac{A}{0.074}$$

If  $P$  plaques are counted, the proportion of nucleated cells producing plaques

$$= \frac{P \times 0.074}{H \times A}$$

And the number of plaque-forming cells per  $10^6$  nucleated cells,

$$N = \left( \frac{7.4 \times 10^4}{A} \right) \times \frac{P}{H} \dots\dots\dots(1).$$

The bracketed part of this expression relates the area of the calibrated high-power field to that of the whole chamber,  $A$ .

1.2. Theoretical expression for variance of N.

Put  $K = \frac{7.4 \times 10^4}{A}$  in equation (1).

Then

$$\text{Var } N = K^2 \left[ (\text{Var } P \cdot \text{Var } \frac{1}{H}) + (\bar{P}^2 \cdot \text{Var } \frac{1}{H}) + (\frac{1}{H})^2 \cdot \text{Var } P \right] \dots (2)$$

(Equation (2) was kindly derived by Dr D. Vere-Jones of Department of Statistics, Australian National University).  $\bar{P}$  represents the mean plaque count, and  $\frac{1}{H}$  the mean of reciprocals of counts of H. The assumption is made that P is the mean number of plaques which would be counted in chambers containing the same number of total lymphoid cells as the chamber considered. So  $P = \text{Var } P = \bar{P}$ . When n counts of H are made,  $\frac{\text{Var } \frac{1}{H}}{n}$  is substituted for  $\text{Var } \frac{1}{H}$ , reflecting the greater accuracy of estimation of  $\frac{1}{H}$ .

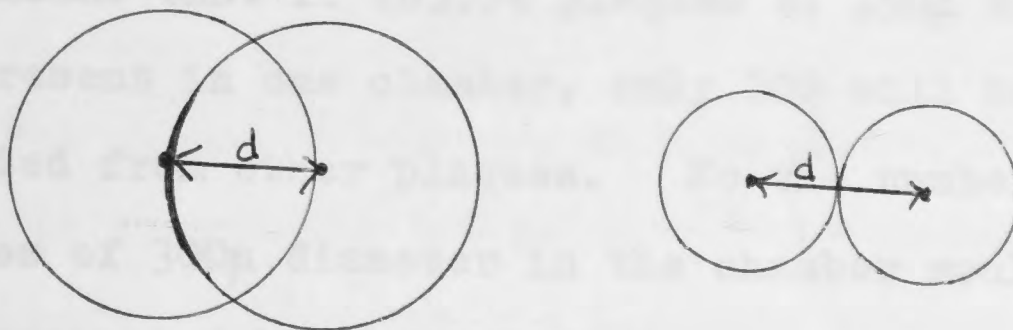
Substituting in (2),

$$\text{Var } N = K^2 P \left[ \frac{1}{n} \cdot \text{Var } \frac{1}{H} (1 + P) + (\frac{1}{H})^2 \right] \dots (3)$$

The variance of  $\frac{1}{H}$  may be derived from variance of H, but this involves calculating third and fourth moments of the distribution of counts of H. It is simpler to work with reciprocals of H.

### 1.3. Errors due to overlap of plaques.

Assume that the maximum extent of overlap of 2 equal plaques such that the two may still be distinguished occurs when the periphery of each passes through the centre of the other. In the case of equal plaques of diameter  $2d$ , the frequency with which overlap greater than this occurs will be the same as the frequency with which plaques of diameter  $d$  will touch or overlap at all (diagram).



From Armitage (1949), the "concentration",  $U$ , of such small plaques in a chamber is given by

$$U = \pi N d^2 / 4 A.$$

where  $N$  = number of plaques

$d$  = diameter of the plaques (assumed all the same)

$A$  = total area of the chamber

**Problem:** In a chamber of area  $100\text{mm}^2$  containing 100 plaques of diameter  $300\mu$ , by how much would the true number of plaques be underestimated? This is equivalent to finding the number of examples of overlap,



however slight, between 100 circles of 150 $\mu$  diameter.

$$\text{Here } U = 100(0.15) / 4 \times 100$$

$$= 0.177.$$

Now the extent of overlapping, m,

$$= \frac{\text{true no. of plaques}}{\text{no. of areas of lysis}},$$

overlapping plaques being taken as one "area of lysis".

To good approximation,  $m = 1 + 2U$

$$= 1.0354.$$

This means that if 103.54 plaques of 150 $\mu$  diameter are present in one chamber, only 100 will be entirely isolated from other plaques. So the number of plaques of 300 $\mu$  diameter in the chamber would be underestimated by about 3.5%.

1.4. In the population of  $54 \times 8 = 432$  spleen segments, 22 contain colonies of cells producing 19S antibody. (It is assumed that all these colonies are located predominantly in only 1 segment). If the 9 7S colonies are allotted at random among the 432 segments, what is the chance that 2 or more of these 7S colonies will "fall" on segments already containing 19S colonies?

The proportion of segments containing 19S colonies =  $22/432 = 0.051$ .

The chance of drawing 0,1,2.....of these segments containing 19S colonies in 9 trials is given by successive terms of the binomial expansion

$$(0.949 + 0.051)^9.$$

The chance that no 7S colony will fall in a segment containing a 19S colony =  ${}^9C_1 (0.949)^9$   
= 0.625

And the chance that 1 7S colony will so fall:

$$= {}^9C_1 (0.949)^8 (0.051)^1$$
$$= 0.290$$

So the chance that 2 or more of these 7S colonies will occur in segments containing 19S colonies:

$$= 1 - (0.625 + 0.290)$$
$$= 0.085$$

APPENDIX 2. (Techniques).

2.1. Preparation of single cell suspensions.

Suspensions of dispersed cells from lymph nodes or spleens were at first obtained by mincing the tissue with scissors in Eagle's medium, then pipetting vigorously. In later experiments, a better initial breakdown of the tissue was achieved by gently rubbing it against a piece of 400-mesh nichrome wire. Large clumps of cells were removed by centrifugation at 120g for a few seconds. The cells in the supernatant were then centrifuged once in Eagle's medium at 240g for 5 minutes. When antibody was present in high concentration in the tissue, a second wash was done. Approximately  $5 - 10 \times 10^7$  dispersed cells (plus occasional small clumps) could be obtained from the spleens of adult CBA mice. From 70 - 90% of these were alive as judged by trypan blue exclusion. About  $10^6$  single cells could be extracted from an unstimulated popliteal lymph node of a mouse.

## 2.2. Lymph and serum antibody titrations.

Samples of lymph or serum were inactivated by heating at 62.5°C for 10 minutes, then diluted in serial 2-fold steps in perspex trays in 0.25ml volumes of 0.9% NaCl solution containing 0.028gm  $\text{CaCl}_2$  and 0.079gm  $\text{MgCl}_2$ / litre. To each well, 0.025ml 5% erythrocytes was added. For haemolysin titrations, unsensitised erythrocytes were used, and for titrations of antibacterial antibody, the red cells were sensitised with the appropriate lipopolysaccharide. For lysis tests, 0.025ml  $\frac{1}{4}$  complement was added to each well, and for agglutination a similar volume of the same batch of guinea-pig serum, which had been inactivated, was used. End points were read as 50% lysis or agglutination.

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